MODULATION OF CALCIUM CURRENTS IN MAMMALIAN CENTRAL SEROTONINERGIC NEURONES

by

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Ne mislim da je ovo umjetnost,
ali ponešto ispadne vrijedno

To My Parents

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Illustrissimo amico salutem! Pro omnia bona et pulchra opera, pro Tua virtute et fidelitate, pro bella memoris, gratias Tibi ago. Ubi amici, ibi opes. Vivat!

ABSTRACT

The dorsal raphé (DR) nucleus, located in mesencephalon, contains 5-hydroxytryptamine (5-HT; serotonin) synthesizing neurones. This putative neurotransmitter is recognised to have a role in many neurological disorders, such as pain, migraine, sleep and anxiety. 5-HT_{1A} receptors, probably located somatodendritically, regulate an autoinhibition of 5-HT release. The effect of phosphorylation on Ca^{2+} current, in the presence and in the absence of 5-HT_{1A} receptor activation, has been studied here using a whole-cell voltage-clamp technique in acutely dissociated adult rat DR neurones.

In various neuronal preparations, transmitters have been shown to reduce voltage-dependent Ca^{2+} currents. In DR neurones, the inhibitory action of 5-HT is incomplete, usually around 60%, and is accompanied by a dramatic slowing of the activation. 8-OH DPAT, a highly specific 5-HT_{1A} agonist, also partially and reversibly reduces high-voltage activated (HVA) Ca^{2+} currents and slows the activation time. It appears that the effect is G-protein mediated and it is believed that the G-protein acts directly on the Ca^{2+} channel proteins, rather than through a freely diffusible second messenger. The action of 8-OH DPAT and GTP-γ-S on both the amplitude and the activation of the current can be relieved by a large depolarising prepulse.

A variety of data indicate that protein phosphorylation regulates the efficacy of synaptic transmission, both by controlling the release of neurotransmitters from the presynaptic nerve terminal and by modulating the sensitivity of receptors in the postsynaptic membrane. Presumably, at rest, some kind of phosphorylation/dephosphorylation balance exists. Evidence has been given that phosphorylation is a necessary step in ion channel functioning. It appears that inhibition of specific phosphatases, could potentiate already activated ion channel currents.

Inhibition of protein phosphatases, by the inclusion of phosphatase inhibitors in the recording pipette, potentiated peak Ca^{2+} current in 80% DR neurones tested, and, in addition, prevented the fast decline or "run-down" of the Ca^{2+} current. Furthermore,

the activation kinetics were speeded up. On the other hand, they produced no change in the inhibition and slowing of the activation of the current caused by the external application of 8-OH DPAT or by G-protein activation. This would suggest that phosphorylation is not directly involved in the effect of neurotransmitters on Ca^{2+} channels. Phosphorylation, however, enhanced the recovery of the activation kinetics in the presence of a prepulse or an increase in temperature.

Peak Ca^{2+} currents are enhanced by inhibition of dephosphorylation and increasing the intracellular cyclic AMP. The effects are additive, together with the enhancements induced by an increase in temperature or a prepulse.

Note: Here I confirm that the work presented is my own and that this Thesis has been composed by myself, in accordance to 3.4.7 regulation of The University of Edinburgh.

Hrvoje Hećimović

CONTENTS

Chapter 1 Introduction

Voltage-activated calcium currents were investigated in this study. In particular, their modulation by the putative neurotransmitter serotonin and the effect of inhibition of dephosphorylation in dorsal raphé neurones were examined. In this introductory section an outline of the proposed classification of calcium channels is given, and effect of neurotransmitters on calcium currents in other cell types is described.

Calcium ions regulate a variety of cellular functions. In neurones, for example, they play a role in diverse events, such as neurotransmitter release, hormone secretion, short- and long-term changes in cell excitability, regulation of Ca-dependent enzymes and gene expression. Calcium channels have been found in all excitable cell types so far studied. Given the diverse nature of their function, it is significant that several types of voltage-gated calcium channels have been described. Development of patch-clamp methods, such as a whole-cell voltage-clamp technique used in this study, has facilitated detailed analysis of the functional and dynamic properties of calcium channels. In addition, parallel biochemical advances have led to insights into their structural properties.

Classification of calcium channels has been proposed on the basis of their biophysical and pharmacological characteristics. More specifically, calcium channels have been differentiated according to voltage-threshold of their activation into high-voltage activated (HVA) and low-voltage activated (LVA) (Llinás & Yarom, 1981; Carbone & Lux, 1984). In addition, it has been shown that the HVA calcium current consists of at least three components that possess specific properties, such as conductance and inactivation rates. N-, L- and P-types are better characterised, however, recent studies have suggested an existence of additional channel types (Fox et al. 1987b; Llinás et al. 1989; Randall et al. 1993; Wheeler et al. 1994). Electrophysiological properties of calcium channels and their presence in different cell types are described in *Chapter 1.1.*

Calcium channels can also be differentiated on the basis of their pharmacological

characteristics. A series of drugs, such as nifedipine and verapamil, used clinically for their antiarrhythmic effects on heart and relaxation of vascular smooth muscle, are also potent blocking agents of calcium channels. For instance, L-type channels are shown to be sensitive to dihydropyridines (Lee & Tsien, 1983; Porzig, 1990). A snail toxin ω-conotoxin VIA is relatively specific blocker of N-type channels, and ω-Aga-IVA blocks P-type current (Kerr & Yoshikami, 1984; Plummer et al. 1989; Mintz et al. 1992). LVA (or T-type) calcium current is resistant to these drugs, but can be blocked by other compounds (Twombly et al. 1988; Akaike et al. 1989). The pharmacological diversity of calcium channels are outlined in *Chapter 1.2.*

The following section of the introduction (*Chapter 1.3*) begins with the analysis of molecular structure of calcium channels. As a result of its high sensitivity to dihydropyridines and its relative abundance in skeletal muscle, L-type calcium channel from this tissue is the best characterised. Its molecular properties with oligomeric structure have been determined (Curtis & Catterall, 1984; Takahashi et al. 1987). Cloning and sequencing of cDNA encoding the α_1-subunit of L-type channel have shown that this subunit probably forms the major functional part of the channel. This was confirmed in the studies of the calcium channel reconstituted into planar lipid bilayers (Curtis & Catterall, 1986).

The fourth section describes some of the postulated functions of calcium channels. It has been proposed that N-type channels regulate transmitter release, L-type channels have a part in secretion, transmitter release and gene regulation, whereas a low-voltage activity of T-type channels suggested their role in cell excitability (Burlhis & Aghajanian, 1987; Hirning et al. 1988a; Hosey & Lazdunski, 1988). In *Chapter 1.4* it is shown that most of the functions are shared by different channel types.

Voltage-dependent calcium channels can be modulated by neurotransmitters, see *Chapter 1.5.* For instance, a whole host of neurotransmitters has been shown to

inhibit calcium currents (Dunlap & Fischbach, 1978; Dolphin et al. 1989; Elmslie, 1992). In many neurones neurotransmitters modulate calcium currents by acting on N-type channels, and in others by a relatively indiscriminate action on several channel types. It has been shown that many receptors are linked to calcium channels by GTP binding proteins (G-proteins), either directly or via second messengers or both (Yatani et al. 1987; Kleuss et al. 1991; Meriney et al. 1994). A role of G-proteins, their different subtypes and coupling with second messengers is described in *Chapter 1.6.*

Serotonin (5-HT) is a putative neurotransmitter with an important role in regulation of various neurologic and non-neurologic states, such as pain, sleep, migraine, anxiety and cardiovascular activities. It is synthesised in dorsal raphé (DR) nucleus, which then sends a large amount of serotoninergic fibres to different parts of central and peripheral nervous system. Previous work (Penington et al. 1991; McAllister-Williams, 1992) that was conducted into the action of 5-HT in DR on both potassium and calcium conductance is presented in *Chapter 1.7.* Effects of serotonin, in particular its modulation of calcium currents are also described here.

Many different processes in cells depend on phosphorylation. It has been suggested that protein phosphorylation modulates synaptic transmission, both by controlling transmitter release and receptor sensitivity. Inhibition of dephosphorylation using specific protein phosphatase blockers has been shown to modulate calcium currents (Hescheler et al. 1988; Frace & Hartzell, 1993). Role of phosphatase inhibitors and changes in phosphorylation/dephosphorylation balance in cells is described in detail in *Chapter 1.8.*

In the closing introductory Chapter, the aims of the investigation of modulation of calcium currents in DR neurones are summarised.

Portrait of calcium channels

Ca channels are transmembrane proteins that allow, in their open conformation, a passive flux of Ca ions across the cell membrane. There is a strong evidence for their existence in both vertebrate and invertebrate cells, for reviews see Reuter (1983), Tsien (1983), Tsien et al. (1988), Bean (1989b), Hess (1990) and Catterall (1993).

One group of Ca channels, called voltage-activated channels, will be described here in detail. They are distinguished from ligand-gated channels, which are divided into receptor-operated channels and second messenger-operated channels (Tsien et al. 1988; Kandel & Schwartz, 1991).

Voltage-sensitive Ca channels are not restricted to classically excitable cells such as neurones, muscle and heart cells, but also exist in non-neuronal cells, such as invertebrate eggs, ciliates, lymphocytes, fibroblasts and GH_3 pituitary cell line (Bean, 1985). They are located in the surface membrane. Many different types of voltage-activated Ca channels have been described, but a generally accepted classification is not yet available. However, a preliminary nomenclature based on their biophysical and pharmacological properties will be used in this presentation. Llinás and Yarom (1981) first pointed to the co-existence of "low-threshold" and "high-threshold" Ca channels, in guinea-pig inferior olivary neurones. Carbone and Lux (1984) demonstrated corresponding low-voltage activated (LVA) and high-voltage activated (HVA) Ca currents in chick dorsal root ganglion (DRG) cells. Single-channel recordings have confirmed this primary difference between the two groups of channels. LVA channels were characterised as T-type (Fox et al. 1987a) and at least three types of unitary conductances for the HVA channels were unveiled (Nowycky et al. 1985; Llinás et al. 1989), known as L-, N- and P-types, see later. Therefore, it seems that at least two major (LVA and HVA) classes of Ca channels exist in central and peripheral neurones. LVA channels exhibit a low threshold of activation (> -50 mV) and inactivate quickly in a voltage-dependent manner. The other, HVA, class

of Ca channels shows a high-threshold of activation (> -20 mV) and inactivate much more slowly.

As described, on the basis of single-channel recordings and using specific channel blockers, three components of the HVA Ca current were characterised, N-, L- and P-type. N- (= neither L nor T or *n*euronal) type Ca channels have been recorded only in cells of neuronal origin. Indeed, it seems that in some neuronal types only N-type Ca currents are present (Tsien et al. 1988; Hess, 1990). N-type channels are activated at potentials positive to -20 mV and blocked irreversibly by the peptide toxin ω-conotoxin GVIA (ω-CgTx) (Fox et al. 1987b; Plummer et al. 1989). This toxin affects L-type current only to a minimal degree (McCleskey et al. 1987; Oyama et al. 1987). Furthermore, N-type channels are insensitive to dihydropyridine (DHP) drugs, unlike L-type channels. They show a unitary conductance from 12 to 15 pS, with 110 mM Ba^{2+} as the charge carrier (Fox et al. 1987a; Swandulla et al. 1991). This type of HVA current has a slower inactivation rate than for T- and faster than for L-type current. The inactivation rate is very variable and, on average, has a time constant, τ_i, of 50-80 ms (Nowycky et al. 1985; Plummer & Hess, 1991). For example, inactivation time constant ranges from around 60 ms in chick DRG neurones (Fox et al. 1987a) to over 500 ms in rat sympathetic neurones (Hirning et al. 1988a), and around 800 ms in neuroblastoma cells (Docherty, 1988). Inactivation time constants in different cells, indeed, can vary from 25 to several hundred milliseconds (Fox et al. 1987b; Gross & Macdonald, 1987; Wanke et al. 1987). Inactivation appears to be dependent on intracellular Ca concentration and duration of depolarisation. The stronger the depolarisation, the more complete the inactivation. However, in some cells even with longer test pulses of several hundred milliseconds, N-type current does not inactivate completely and a large sustained, slowly inactivating component is present (Plummer & Hess, 1991). There even appears to be diversity of inactivation time constants of N-type current within a single cell type. In frog sympathetic neurones, N-type channels can be seen that rapidly inactivate (τ_i=

50 ms) with depolarisations to -10 mV in some patches, while in others there appears to be little or no inactivation during a 320 ms test pulse (Kongsamut et al. 1989). Other reports also described the inactivation rates of individual, otherwise identical, channels to vary by more than a factor of ten (Cavalié et al. 1985; Rittenhouse & Hess, 1994). Single channel recordings of N-type channel openings in rat superior cervical ganglion neurones have suggested that not only can different types of N-type current inactivation be seen in a single cell, but individual channels may be able to switch between slow and fast inactivating states (Plummer & Hess, 1991).

L- (= *l*ong lasting) type of Ca channels can be operationally defined at the single channel level by a combination of HVA voltage-dependence, slow or negligible inactivation, large unitary slope conductance (20-27 pS), responsiveness to BayK 8644 and other DHP drugs and slightly larger permeability for Ba^{2+} than for Ca^{2+} ions (Fox et al. 1987b; Bean, 1989b; Yang et al. 1993; Haack & Rosenberg, 1994). The channels activate at relatively positive test potentials (> -10 mV) and show little inactivation during depolarisation. The L-type current can be isolated at a holding potential of -40 mV, where N- and T-type currents are inactivated. Openings of L-type Ca channels are greatly enhanced by BayK 8644, a DHP Ca channel agonist, whereas N-type and T-type Ca channels are not significantly affected (Fox et al. 1987a). However, there are some variations present among different cell types; activation and inactivation kinetics may vary widely and, furthermore, it appears that a small sensitivity to ω-CgTx exists, at least in some neuronal cells, see *Chapter 1.2.* DHP-sensitive L-type Ca channels are found in most neurones, gland and muscle cells (Bean, 1989b; Pelzer et al. 1990; Porzig, 1990).

Some HVA Ca channels possess different conductances and inactivation rates than N- and L-type channels and they are ω-CgTx- and DHP-insensitive (Charlton & Augustine, 1990). Llinás et al. (1989) discovered a novel P-type channel type in cerebellar *P*urkinje cells. The channel exhibits 5-8 pS conductance in 100 mM Ca^{2+}

as the charge carrier. The P-type current shows a very slow rate of inactivation during a 2 s test pulse, and in that way is similar to L-type current (Llinás et al. 1989). However, P-type channels are inactivated at holding potentials between -60 and -20 mV (Regan, 1991), which is considerably more negative than for L-type channels (Fox et al. 1987b). Furthermore, P-type channel is sensitive to Cd^{2+} and Co^{2+}, but not to ω-CgTx or DHPs (Llinás et al. 1989). The specific block of the channel was achieved by an application of a venom toxin FTX. In cerebellar slices, FTX blocked Ca-dependent spikes and in a squid giant synapse inhibited synaptic transmission without affecting the presynaptic action potential. A synthetic P-type channel blocker ω-Aga-IVA has proved to be a useful tool in investigations of putative P-type channels in different cell types (Mintz & Bean, 1993). So far, P-type channels have been found to be abundant in cerebellar (Pearson et al. 1993; Momiyama & Takahashi, 1994) and spinal neurones (Momiyama & Takahashi, 1994), hippocampal CA1 region (Mintz & Bean, 1993; Momiyama & Takahashi, 1994), rat visual cortex (Mintz & Bean, 1993), rat neocortical pyramidal neurones (Brown et al. 1994), DRG cells (Mintz & Bean, 1993), in a smaller quantity in hippocampal CA3 area (Mintz & Bean, 1993) while none were found in sympathetic ganglion neurones (Mintz & Bean, 1993).

Recently, it has been suggested that some other (sub)types of HVA Ca channels exist. Randall et al. (1993) isolated a current that was insensitive to specific channel blockers, but was inhibited by Cd^{2+}, ω-CgTx-MVIIC and by a large concentration of ω-Aga-IVA. In cerebellar granule cells, this novel Q-type Ca current constitutes 43% of total Ca current. This is a HVA current and possibly has a role in synaptic transmission, together with N-type current (Wheeler et al. 1994). It is thought that another recently described R-type HVA Ca current also exists in cerebellar granule cells (Wheeler et al. 1994). The R-type current appears to be sensitive to Cd^{2+} and Ni^{2+} and it is inhibited by ω-CgTx-GVIA very slowly, unlike N-type current (Ellinor et al. 1993).

It appears that a combination of ω-CgTx and DHP antagonists completely blocks Ca current in many cell types, such as neuroblastoma cells (Narahashi et al. 1987), but not in all. Various cells, such as rat pheochromocytoma cells (Plummer et al. 1989), DRG neurones (Regan et al. 1991; Scroggs & Fox, 1992a), hippocampal cells (Mogul & Fox, 1991; Regan et al. 1991), spinal cord neurones, visual cortex (Regan et al. 1991) and rat dorsal raphé neurones (Penington et al. 1991) contain other, yet unidentified, type of HVA current. It is possible that a part of it is P-type current, because FTX has not yet been applied to all of these cells. However, single-channel experiments showed that this unidentified type of HVA current might exhibit, unlike P-type current, similar unitary conductance to N- and L-type current.

LVA Ca channels such as T-type channels, have been described in a variety of cells. There is evidence that suggests their presence in neuronal, glandular, heart and skeletal muscle cells (Fox et al. 1987b; Liu & Lasater, 1994b). In addition to chick (Carbone & Lux, 1984; Carbone & Lux, 1987; Fox et al. 1987a; Swandulla et al. 1991), rat (Ewald et al. 1988a; Mintz et al. 1992; Scroggs & Fox, 1992b) and mouse (Anderson & Harvey, 1987; Kostyuk & Shirokov, 1989) DRG neurones, T-type has also been identified in mammalian hippocampus (Tsien et al. 1988), neocortex (Brown et al. 1994), neostriatum (Bargas et al. 1991; Hoehn et al. 1993; Surmeier et al. 1993), retinal ganglion cells (Liu & Lasater, 1994b), spinal motoneurones (Berger & Takahashi, 1990), cerebellar Purkinje cells (Regan, 1991; Regan et al. 1991; Mintz et al. 1992; Bindokas et al. 1993; Hockberger & Nam, 1994), amygdala (Zhou et al. 1994), dorsal raphé (Penington & Kelly, 1990; Penington et al. 1991) and neuroblastoma cells (Tsunoo et al. 1985; Williams et al. 1985; Narahashi et al. 1987). There are no such apparent differences in T- (= *t*ransient) type channels in different cell types. They share a small unitary conductance (5-8 pS), rapidly transient channel kinetics, slow deactivation of tail currents, activation at more negative potentials (> -60 mV) and stability of the channel activity in cell-free conditions (Carbone & Lux, 1984; Fox et al. 1987b; Bean, 1989b; Porzig, 1990; Schroeder et al. 1990). Unlike

N- and L-type channels, T-type channels have equal unitary conductance for Ca^{2+} and Ba^{2+} (Porzig, 1990). Electrophysiologically, T-type channels activate at more negative potentials than the other channel types and can thus be studied in relative isolation. This is also a some what variable aspect of T-type channels, because the voltage-dependency of activation of T-type channels can become significant at potentials as negative as -65 mV in sensory neurones (Carbone & Lux, 1984; Fox et al. 1987b), but at -50 mV in heart cells (Bean, 1985). In DRG cells, the current amplitude and the rate of decay of the current increases with stronger depolarisations until both reach a maximum at approximately -40 mV (Carbone & Lux, 1984). Scroggs and Fox (1991; 1992a) proposed that the density of T-type current in neurones could depend on the cell size. In DRG neurones, T-type current was more prominent in smaller rather than larger diameter neurones. T-type channels are more sensitive to Ni^{2+} and amiloride and, in general, they are resistant to DHP drugs, although some reports suggested that these compounds could non-specifically block T-type current (Bean, 1989b; Pelzer et al. 1990). They are more resistant to block by Cd^{2+} ions than L-type channels and block by ω-CgTx is very weak and reversible (Nowycky et al. 1985; Fox et al. 1987b). Because of the lack of specific ligands, biochemical analyses of these channels have been sparse.

Not all cells possess both classes of Ca channels. HVA and LVA channels co-exist in central neurones, such as hippocampal pyramidal CA3 cells (Mogul & Fox, 1991), rat petrose ganglion neurones, rat, mouse and chick DRG neurones and mouse cerebellar granule cells (Slesinger & Lansman, 1991a). An increase in the proportion of HVA current with ontogenetic development has been described in hippocampus, chick DRG cells and autonomic neuronal precursor cells (Yaari et al. 1987; Bean, 1989b; Thompson & Wong, 1991). N- and L- but virtually no T-type channels are expressed in frog sympathetic neurones (Lipscombe & Tsien, 1987), rat myenteric plexus neurones (Hirning et al. 1990) and superior cervical ganglion cells (Hirning et al. 1988a). T- and L- but no N-type are reported in chick and newborn rat DRG

neurones, rat hippocampal CA1 cells, rat petrose and nodose neurones, neuroblastoma (N1E-115) cells, GH_3 pituitary cells, cardiomyocytes from dog and frog atrium, most muscle cells, gland cells, ciliates, fibroblasts and astrocytes (Tsien et al. 1988). Some cells appear to express only one type of HVA channels, e.g. L-type in adrenal chromaffin cells (Fenwick et al. 1982; but see Artalejo et al. 1994). It was suggested that T-type current is never found in the absence of HVA current, however there are some exceptions, as in neoplastic B lymphocytes, where only T-type channels are present.

N-, L- and T-type channels can be further characterised by their ion selectivity and sensitivity to inorganic blockers. N- and L-type channels are more permeable to Ba^{2+} than Ca^{2+}, whereas T-type channels show equal selectivity for these two cations in sensory, neuroblastoma, hippocampal and cerebellar granule cells (Fox et al. 1987b; Yaari et al. 1987; Kostyuk & Shirokov, 1989; Marchetti & Robello, 1989; Slesinger & Lansman, 1991a; Kasai, 1992). However, it has been reported that in rat hypothalamic neurones, substituting Ba^{2+} for Ca^{2+} decreases LVA current by 40-50% (Akaike et al. 1989). One explanation could be that these two ions have a differential effect on Ca channel gating. Indeed, it was shown that less inactivation of HVA current was observed in the presence of Ba^{2+} rather than Ca^{2+} (Eckert & Chad, 1984; Sanguinetti & Kass, 1984; Kass, 1987). Specific inorganic drugs could also help to differentiate Ca channel currents in most cell types. L- and N-type channels appear to be more sensitive to Cd^{2+}, whereas T-type channel is easily blocked by Ni^{2+} (Fox et al. 1987b; Yang et al. 1993). In chick and mouse DRG neurones, 20-50 μM Cd^{2+} blocked 90% of HVA current and 50% of T-type current (Fox et al. 1987b; Gross & Macdonald, 1987). However, in rat hippocampal cells, Cd^{2+} completely suppressed both the HVA and LVA components (Yang et al. 1993). In cerebellar granule cells, Cd^{2+} (1 μM) completely abolished all calcium currents (Bossu et al. 1994). Ni^{2+} was more specific in its block and rather selectively reduced T-type current but not HVA current (Fox et al. 1987b). Similar observations have been made in neuroblastoma

cells (Narahashi et al. 1987; Twombly et al. 1988) and hippocampal cells (Mogul & Fox, 1991). However, Akaike et al. (1989) reported that LVA current was more sensitive to Cd^{2+} than Ni^{2+} in hypothalamic neurones, and the current was also sensitive to Zn^{2+}. Sensitivity of the LVA channel to Zn^{2+} has also been demonstrated in amygdaloid neurones, where these currents are more sensitive to Ni^{2+} than to Cd^{2+} (Akaike et al. 1989). The trivalent cation La^{3+} appears to be non-selective in the inhibition of Ca currents and blocks both HVA and LVA components in mouse DRG (Kostyuk & Shirokov, 1989), rat hippocampal, amygdaloid and hypothalamic neurones (Akaike et al. 1989). Another lanthanide Gd^{3+} blocks predominantly N-type current with a presumably direct action on the channel, in neuroblastoma-glioma hybrid cell line NG108-15 (Docherty, 1988).

None of the channel types have been identified exclusively with one particular biological role. However, some evidence suggests that they might have specific functions. T-type channels are often portrayed as generators of pacemaker activity while L- and N-type channels could have a role in secretion and release of neurotransmitters (Tsien et al. 1988). T-type channels have been shown to contribute to pacemaker activity in heart cells (Hagiwara et al. 1988) and in mammalian CNS neurones (Burlhis & Aghajanian, 1987). Ca entry through L-type channels has been shown to link excitation to a variety of different responses: contraction in heart (Bean, 1985), expression of *c-fos* in PC-12 cells (Morgan & Curran, 1986; Sheng & Greenberg, 1990), release of substance P from DRG neurones (Rane et al. 1987) and release of norepinephrine (NE) from undifferentiated PC-12 cells (Kongsamut & Miller, 1986). N-type channels seem to play a dominant role in controlling transmitter release in certain cases, e.g. NE release from sympathetic neurones, which is DHP-resistant, but sensitive to both Cd^{2+} and ω-CgTx (Hirning et al. 1988a). Wheeler et al. (1994) argued that synaptic transmission between hippocampal CA3 and CA1 neurones was mediated by N-type channels. Selective inhibition of N-type Ca currents by neurotransmitters and neuromodulators in a number of cells is also

consistent with the blocking of synaptic function found in other studies, see *Chapter 1.5.*

Localization of a specific Ca channel type to a particular region of a neurone has been proposed. There is evidence that N- and L-type Ca channels co-exist in motor nerve terminals (Tsien et al. 1988; Stanley, 1991; Farilas et al. 1993) and in cell bodies of sympathetic neurones (Lipscombe et al. 1988b; Delcour & Tsien, 1993; Golard & Siegelbaum, 1993). The channels are sometimes found clustered in "hot-spots" (Smith & Augustine, 1988). Proximity of Ca channel "hot-spots" to Ca-sensitive mechanisms may be important for the rapid triggering of physiological processes such as transmitter release in synaptic terminals (Tsien et al. 1988; Zucker, 1993a; Zucker, 1993b).

No interconversion between channel types has been detected (Fox et al. 1987a). It appears that they are different but closely related proteins. A similar conclusion has been reached in lipid bilayers studies of multiple Ca channels with different conductances, kinetics and pharmacology. However, this hypothesis still awaits direct structural analysis.

Pharmacology of calcium channels

As has been described in the previous chapter, there is still no generally accepted classification of Ca channels. However, the existence of different types of Ca currents could be studied on the basis of their voltage-dependency and different pharmacological properties. The drugs that bind to Ca channels can, in general, be described as Ca channel blockers or channel activators. In experimental and clinical work, the most often used are 1,4-dihydropyridine derivatives (BayK 8644, nimodipine, nifedipine and nitrendipine), phenylalkylamines (verapamil, D 600 and

D 888) and benzothiazepines, e.g. diltiazem. Some other compounds also affect Ca channel currents, such as ω-CgTx, diphenylpiperazines (cinarizine, flunarizine) and diphenylbutylpiperazines (pimozide, fluspirilene).

N-type Ca channels are rather specifically blocked by the peptide toxin from a venom of the marine snail *Conus geographus*, ω-CgTx, but not by DHP antagonists. L-type channels are shown to be sensitive to DHPs and to various other organic compounds. In skeletal muscle, heart and smooth muscle, L-type channels are insensitive to ω-CgTx, whereas it is possible that similar channels from neuronal tissue are slightly affected by the toxin (Porzig, 1990). Finally, T-type channels can be blocked by phenytoin and amiloride and they are essentially resistant to DHPs and to ω-CgTx, for reviews see Godfraind et al. (1986), Tsien et al. (1988), Bean (1989b), Hess (1990), Pelzer et al. (1990), Porzig (1990) and Swandulla et al. (1991).

The best characterised are L-type channels, that were first described in mammalian cardiac cells on the basis of their electrophysiological properties (Reuter et al. 1982; Cavalié et al. 1983). Later, it was shown that the channel could be specifically blocked by 1,4-dihydropyridine antagonists and other drugs (Lee & Tsien, 1983). DHP analogues have not been shown to be effective in all cell types. At concentrations of 10 μM or more they do not modify Ca spikes in a variety of cells, such as rat pars intermedia, rat synaptosomes and cortical slices, guinea-pig cortex and hippocampus (Ogura & Takahashi, 1984). Nifedipine at the concentration of 10 μM had no effect on Ca current in rat superior cervical ganglion cells (Gurney et al. 1985). Furthermore, phenylalkylamines and benzothiazepines block Ca current only at higher concentrations (10-100 μM), as in rabbit nodose ganglion cells (Kurachi et al. 1989). This has led to suggestions that their action is non-specific. However, Ca channel blockers, and in particular DHP compounds, have been shown to reduce the L-type component of Ca current in many cell types (García et al. 1984; Sanguinetti & Kass, 1984; Rane et al. 1987; Akaike et al. 1989; Aosaki & Kasai, 1989; Scott & Dolphin, 1990). Single-channel recordings showed that the DHP antagonist nifedipine

decreased L-type channel opening probability in chick (Fox et al. 1987b) and in mouse (Kostyuk & Shirokov, 1989) sensory neurones. It appears that the block by DHP antagonists of the current is stronger in cardiac or smooth muscle cells than in neurones. Bean (1989b) showed that the block is typically less than 50% of the total current in DRG cells, whereas 100% in muscle. In neurones the block by DHPs is never complete. Fox et al. (1987b) showed that nifedipine (10 μM) blocked 60% of L-type current evoked from V_H -40 mV, while producing no effect on current elicited from V_H -80 mV in chick DRG cells. On the other hand the same concentration of the drug completely abolished L-type current in cardiomyocytes (Sanguinetti & Kass, 1984). This finding suggests that neuronal L-type channels have lower sensitivity to DHPs than muscle L-type channels or that the elicited current was not completely L-type current. Furthermore, some neurones that possess L-type channels are completely insensitive to DHP compounds. Nifedipine and BayK 8644 did not affect Ca current in hippocampal, cortical (Ogura & Takahashi, 1984) and rat sympathetic (Gurney et al. 1985) neurones.

Drugs with a stimulating effect on L-type Ca channel activity, like BayK 8644, 202-791, H 160/51, have only been discovered among the DHP derivatives (García et al. 1984; Tiaho et al. 1990). The DHP agonist BayK 8644 (5 μM) enhanced the activity of L-type Ca channels, but not that of N- or T-type channels (Nowycky et al. 1985; Fox et al. 1987b). The tail current, which is the most consistent part of L-type current, is found to be greatly prolonged by an application of DHP agonists following repolarisation (Brown et al. 1983; Plummer et al. 1989; Cox & Dunlap, 1992). In electrophysiological studies, an increase in inward Ca current by Ca channel activators was mainly due to a marked prolongation of the single-channel open state (Brown et al. 1984; Fox et al. 1987a). Single-channel studies in DRG neurones and rat hippocampus suggested that nifedipine (10 μM) caused fewer L-type channel openings, and the application of BayK 8644 (5 μM) left the channels in the open state for longer (Rosenberg et al. 1986; Fox et al. 1987a; Kostyuk & Shirokov, 1989;

Mogul & Fox, 1991). BayK 8644 (5 μM) increased Ca current amplitudes in variety of cells, including neuroblastoma (Docherty, 1988) and DRG (Fox et al. 1987b; Scott & Dolphin, 1988) cells. However, it appears that DHP agonists can not open Ca channels alone, i.e. in a voltage-independent manner. Furthermore, one group of stereoisomers acted as activators, e.g. (-)BayK 8644 and (+)202-791, and the other group as blockers, e.g. (+)BayK 8644 and (-)202-791 (5 μM) of the channel in cultured rat sensory neurones (Dolphin & Scott, 1988). The effect of both the activators and the blockers was voltage-dependent. Ca currents were potentiated only when elicited from fairly negative holding potentials (V_H -80 mV); at more positive holding potentials, for instance above -30 mV, an inhibition of the currents rather than an activation was observed. This effect was not seen in guinea-pig ventricular cells (Hamilton et al. 1987), suggesting that it might be tissue dependent.

Most authors agree that these activators and blockers share a common high-affinity DHP binding site in membrane (Williams et al. 1985; Hamilton et al. 1987). Some toxins, like gonioporatoxin, atrotoxin and taicatoxin (Porzig, 1990) have also been shown to interact allosterically with this DHP binding site. Gonioporatoxin and atrotoxin are both activators, and taicatoxin is a voltage-dependent blocker of L-type channels.

There is evidence (Porzig, 1990) to suggest that L-type Ca channels could also be modulated indirectly, via a specific intracellular mechanisms. The author argued that three mechanisms are predominant. The first one describes changes in receptor density or affinity. For example, in frog ventricular cells, β-adrenergic stimulation causes a large increase in L-type Ca current, thought to be due, at least in part, to a recruitment of, at rest, "unavailable" channels (Bean, 1985). cAMP- and/or Ca-mediated phosphorylation probably play a role here. The other two modulatory mechanisms characterise an involvement of G-proteins and the effect of phosphorylation of the channel protein by endogenous protein kinases. It has been suggested that the effect of DHP Ca channel activators like BayK 8644, at least in

neurones, critically depends on the presence of activated G-proteins (Scott & Dolphin, 1988). This may explain the rather unusual observation that when rat DRG neurones are pretreated with pertussis toxin (PTX), BayK 8644 causes a decrease in Ca current, rather than an increase. It therefore appears that BayK 8644 requires a PTX sensitive G-protein to be able to elicit activation of Ca current. When the G-protein is inactivated, only inhibition of the current is seen. Furthermore, G_s-mediated amplification of the stimulatory effect of BayK 8644 has been observed in myocardial Ca channels (Yatani et al. 1987; Yatani et al. 1988). These results suggest that G-proteins help to maintain the Ca channel protein in an activatable state, perhaps by stabilizing the resting or open state of the channel, see *Chapter 1.6.* Therefore, it was to expect that G-proteins would antagonise the effect of Ca channel blockers, which appear to act by stabilizing the inactivated channel state. Indeed, Scott and Dolphin (1987) reported a loss of blocking potency for DHP antagonists, verapamil and d-cis-diltiazem in GTP-γ-S perfused DRG neurones.

Binding studies using tissue homogenates from heart, smooth muscle, skeletal muscle and brain have shown that DHPs, phenylalkylamines and benzothiazepines, that all act on L-type channels, occupy distinct but allosterically coupled binding sites on the channel protein, probably located on the non-glycosylated α_1-subunit of the voltage-gated L-type channel (Godfraind et al. 1986; Janis et al. 1987; Hosey & Lazdunski, 1988). Catterall (1993) suggested that the effect of DHPs occurs some distance away from the DHP binding site, and that DHPs are probably allosteric modulators of L-type channel rather than simple channel blockers. One needs to be cautious in the interpretation of the results of DHP blockers. It might still be possible that the compounds affect either non-inactivating N-type component of HVA current or some other, yet unidentified, neither L- nor N-type, Ca current.

N-type Ca channel current, with inactivation rates slower than in T- and faster than in L-type currents, has been described using single-channel patch-clamp recordings

in neuronal cells (Fox et al. 1987b). The inactivation time constant covers a broad spectrum (50-500 ms) with conductances of 11-20 pS (Tsien et al. 1988), see *Chapter 1.1.* Therefore, it appears that the electrophysiological criteria are not sufficient to characterize this type of current. An important finding was that this neuronal Ca channel can be blocked by the snail venom ω-CgTx (Kerr & Yoshikami, 1984; Oyama et al. 1987; Farilas et al. 1993). Many researchers (Aosaki & Kasai, 1989; Plummer et al. 1989; Bossu et al. 1991; Regan et al. 1991; Scroggs & Fox, 1991; Cox & Dunlap, 1992) showed that ω-CgTx blocks N-, but not L-type channels. Previously, it was thought that ω-CgTx blocked both N- and L-components of HVA current. This was argued by some groups working on chick DRG cells (Kerr & Yoshikami, 1984), rat sympathetic ganglion and in hippocampal (McCleskey et al. 1987) neurones. But, when DHP antagonists became available to identify selectively the current component exclusively carried by L-type channels, ω-CgTx was shown to be unable to block L-type current in frog and rat sympathetic ganglion neurones (Plummer et al. 1989). In rat pheochromocytoma PC12 cells, L-type current appears insensitive, but ω-CgTx reveals two pharmacologically distinct components of N-type current. One is blocked irreversibly, and the other reversibly (Plummer et al. 1989). In agreement with that hypothesis, Kasai and Aosaki (1989) found no evidence for persistent block of L-type channels by ω-CgTx in chick DRG neurones. That was later confirmed by Aosaki and Kasai (1989) at the single channel level. Therefore, it appears that ω-CgTx could be used to analyse the N-type component of Ca current (Bean, 1989b; Cox & Dunlap, 1992), although L-type channels in neurones might be slightly sensitive to the toxin (Porzig, 1990). This is additionally supported by the fact that ω-CgTx has little or no effect on the Ca currents in cardiac, smooth or skeletal muscle (McCleskey et al. 1987) that do not contain N-type channels. It was also suggested that the effect of ω-CgTx is stronger in peripheral neurones, where 70-90% of the total current is blocked by this toxin, than in central neurones with 50-60% block (Protti et al. 1991; Swandulla et al. 1991). In addition to that, ω-CgTx, but not DHP antagonists, strongly inhibit neurotransmitter release in central neurones

(Kerr & Yoshikami, 1984).

Evidence for an indirect modulation of N-type channels is also available. Specific inhibition of N-type Ca current by transmitters was observed, as with dynorphin A acting on κ-opioid receptors in mouse DRG cells (Werz & Macdonald, 1985; Gross & Macdonald, 1987), neuropeptide Y in myenteric plexus (Hirning et al. 1988a), adenosine in hippocampal neurones (Madison et al. 1987) and noradrenaline acting on α-adrenoreceptors in frog sympathetic ganglia (Lipscombe et al. 1988b), see *Chapter 1.5.*

Llinás et al. (1989) described P-type HVA channel, that is neither DHP nor ω-CgTx sensitive and it is blocked by a toxin fraction from a funnel-web spider *Agelenopsis aperta* venom - FTX. This channel has recently been cloned and the lack of DHP and ω-CgTx sensitivity confirmed (Mori et al. 1991). Originally it was found in cerebellar Purkinje cells to constitute 90% of the HVA Ca current, and using a synthetic P-type channel blocker ω-Aga-IVA, the current was shown to exist in other cell types. In hippocampal CA1 neurones the P-type current component is 26% of the total Ca current, in visual cortex it constitutes 32%, spinal cord 45%, DRG neurones 23% and hippocampal CA3 neurones 14% (Mintz et al. 1992). The block of P-type current is voltage-dependent. The current is blocked by Cd^{2+} and Co^{2+} and showed an unusual sensitivity to block by Ni^{2+} ions (Llinás et al. 1989).

The Q- and R-type channels in cerebellar granule neurones are resistant to blockade by ω-CgTx-GVIA, nimodipine and ω-Aga-IVA at concentrations sufficient to eliminate N-, L-, and P-type channels, respectively (Wheeler et al. 1994). The Q-type channels are completely blocked by ω-CgTx-MVIIC and by a high concentration of ω-Aga-IVA (100 times larger concentration than needed for the P-type channel block). In contrast, R-type channels are little affected by either of these treatments. The current is slowly inhibited by ω-CgTx-GVIA, unlike N-type current (McCleskey et al. 1987; Aosaki & Kasai, 1989).

A low-threshold, rapidly inactivating T-type Ca current has been shown in many cells containing voltage-dependent Ca channels (Tsien et al. 1988). However, there is a lack of reports on specific modulation of this Ca current component by drugs and neurotransmitters. The relevant observations from electrophysiological recordings using a voltage-clamp or single-channel technique were able to distinguish T-type current from L-type on the basis of voltage-dependency. Biochemical analysis showed that T-type channel is a separate identity that consists of specific proteins, which are different than for N- or L-type channels and also the product of different genes. For instance, in myodysgenic mice, a mutant that lacks L-type Ca channels in skeletal muscle cells, T-type currents are fully preserved (Bean, 1985). In spite of a lack of a high-affinity ligand that could be used to analyse T-type channels, some drugs inhibit T-type currents preferentially and have only low potency for L-type channels. Phenytoin blocks transient T-type Ca current in neuroblastoma cells at concentrations of 3-100 μM, not affecting the slowly inactivating L-type component (Twombly et al. 1988). Flunarizine is also shown to be a potent blocker of LVA current in rat hypothalamic neurones (Akaike et al. 1989). The same group reported an interesting finding that BayK 8644 partially blocked LVA current in hypothalamus. Other examples include retinoic acid and tetramethrin (Tsunoo et al. 1985) and the diuretic compound amiloride (Tang et al. 1988; Mogul & Fox, 1991). Among the inorganic cations, Ni^{2+} in low concentrations of 40 μM blocked T-type current in rabbit sinoatrial node cells (Hagiwara et al. 1988) and in chick sensory neurones (Fox et al. 1987b). Cd^{2+} and Co^{2+} also blocked T-type channel (Hagiwara et al. 1988; Schroeder et al. 1990).

Indirect inhibition of T-type currents, probably via G-protein mediated mechanisms, has also been reported (Berger & Takahashi, 1990; Schroeder et al. 1990; Scott et al. 1990; Liu & Lasater, 1994a). In neuronal cells, a functional coupling of T-type channels to G-protein (possibly G_o) is suggested in studies with the putative neurotransmitter neuropeptide Y. The drug inhibited T- and L-type currents in rat DRG cells (Ewald et al. 1988b). In both cases PTX abolished this inhibitory response.

Marchetti et al. (1986) showed that dopamine irreversibly inhibited T-type current in chick DRG neurones. In the same experiment, HVA current showed a small and reversible slowing of the activation kinetics.

Structure of calcium channels

Voltage-sensitive Ca channels constitute an essential link between transient changes in membrane potential and a variety of cellular responses including secretion of neurotransmitters and hormones, initiation of contraction in cardiac and smooth muscle, and activation of second messenger responses in many cell types.

The L-type Ca channel is uniquely sensitive to DHP drugs and due to the high density of DHP binding sites on Ca channels in skeletal muscle, these are the most extensively studied and characterised, for reviews see Brown et al. (1984), Janis et al. (1987), Campbell et al. (1988), Hosey & Lazdunski (1988), Hess (1990) and Catterall (1993).

The reports (Curtis & Catterall, 1984; Borsotto et al. 1985; Catterall, 1988; Catterall, 1993; Zhang et al. 1993; Bean, 1994b) describing the structure of a purified Ca channel from skeletal muscle, showed the existence of an α-subunit (170 kDa), a β-subunit (50 kDa), a γ-subunit (32 kDa) and δ-subunit (26 kDa). All subunits constitute integral components of the DHP receptor. The hypothesis that the DHP receptor is composed of these subunits has been confirmed by reconstitution in lipid bilayers. The reconstituted channel possessed the binding sites for Ca channel blockers (Curtis & Catterall, 1986).

It appears that the α-subunit of the DHP-sensitive Ca channel is composed of two distinct components, the α_1- and the α_2-subunit (Tanabe et al. 1987; Campbell et al. 1988; Ellis et al. 1988). The α_1-subunit probably contains the binding sites for dihydropyridine and phenylalkylamine (Hosey & Lazdunski, 1988; Hess, 1990) and is also a substrate for cAMP-dependent protein kinase (Hosey et al. 1986; Hosey & Lazdunski, 1988), Ca/calmodulin-dependent protein kinase (Hosey et al. 1986; Takahashi et al. 1987) and protein kinase C (Campbell et al. 1988). The primary amino acid sequence of the α_1-subunit has been deduced from its cDNA and it was shown to contain four domains of high homology and each domain is believed to possess six, presumably α-helical, membrane-spanning segments (S1-S6) (Tanabe et al. 1987). The S4 segment contains a pattern of positive charges and appears to be a voltage sensor of the Ca channel (Tanabe et al. 1987). The DHP binding site is located at the interface between domains S3 and S4, and may affect domain-domain interactions that are important determinants of activation gating (Catterall et al. 1993). Kinetic properties, such as inactivation kinetics, appear to be determined by a sequence of amino acids in the S6 segment of the first repeat of the α_1-subunit (Zhang et al. 1994). Furthermore, the α_1-subunit has a higher degree of homology to the α-subunit of the voltage-activated Na^+ channel (Tanabe et al. 1987; Williams et al. 1992).

The α_2-subunit and the δ-subunit form an $\alpha_2\delta$ complex. The α_2-subunit is easily identified following treatment of $\alpha_2\delta$ complex with sulfhydryl reducing agents. When treated with sulfhydryl reducing agents this complex undergoes a reduction in molecular mass. This is probably due to the dissociation of an additional protein component, the δ-subunit (Takahashi et al. 1987). It has been reported that the α_2-subunit consists of a large glycosylated extracellular domain and a relatively small transmembrane domain (Takahashi et al. 1987; Williams et al. 1992). This subunit is not phosphorylated and does not contain binding sites for DHP or phenylalkylamine Ca channel blockers. The functional role of the α_2-subunit is still

unclear.

The δ-subunit contains a hydrophobic transmembrane domain. The functional role of the δ-subunit is not known.

The β-subunit of the DHP-sensitive Ca channel has been shown to be a substrate for cAMP-dependent protein kinase and protein kinase C (Curtis & Catterall, 1986; Ishikawa et al. 1993). It is tightly associated with an intracellular domain of α_1 and possibly facilitates coupling between movement of the voltage sensors and opening of the channel pore (Bean, 1994b). A monoclonal antibody to the β-subunit affects the current magnitude and activation and inactivation kinetics of the Ca channel reconstituted into planar lipid bilayers (Hymel et al. 1988). This result and the presence of phosphorylation sites on the β-subunit, suggest that the peptide may play a part in regulation of Ca channel activity.

There is evidence to suggest that the γ-subunit has both extracellular and transmembrane domains and, so far, is shown to be unique to skeletal muscle (Curtis & Catterall, 1984; Borsotto et al. 1985; Campbell et al. 1988). The γ-subunit appears to be a tightly associated regulatory subunit of the Ca channel.

The oligomeric subunit structure of the skeletal muscle channel, shows that the α_1-subunit is the major transmembrane component of the receptor and the α_2-subunit is the major glycoprotein. The β-subunit is mostly cytoplasmic protein, but may be tightly associated to the membrane or other subunits. Finally, the γ-subunit is a part of the membrane and is a glycoprotein. The association of the four subunits appears to be very tight and highly specific (Campbell et al. 1988). Takahashi et al. (1987) proposed a model for the subunit structure of the DHP-sensitive Ca channel in skeletal muscle, see Figure 1.1, in which the α_1-subunit forms the ion channel and is surrounded by the α_2- and γ-subunits. The β-subunit is probably attached non-covalently to the cytoplasmic side of α_1, whereas the δ-subunit forms a complex with

the α_2-subunit. The $\alpha_2\delta$ complex extends to the external surface and contains glycosylation sites. Similar model has also been suggested by other researchers (Catterall, 1988; Hosey & Lazdunski, 1988).

It is still not clear whether other Ca channels are similar to the skeletal DHP sensitive channel. Antibodies raised against the S3 - S4 loop of the α_1-subunit of the DHP sensitive skeletal muscle channels cross react with DHP insensitive channels in neuroblastoma cells (Reeve et al. 1994). Nevertheless, mice that lack skeletal muscle DHP receptors, have normal levels of DHP binding sites in heart and sensory neurones (Tanabe et al. 1991; Mori et al. 1993; Tanabe et al. 1993), which is an indication that there are differences between DHP sensitive channels in different regions. In addition, the unitary conductance of skeletal DHP channels is lower and they activate around ten times slower than DHP channels elsewhere (Rosenberg et al. 1986). It is possible that different genes code for the channels in different tissues. There are certain similarities with recently cloned human neuronal L-type channel. This channel was shown to possess most of the predicted voltage and pharmacological properties (Williams et al. 1992). Interestingly, this group reported, that the α_1-subunit does not pass current in the absence of the β-subunit.

Functions of calcium channels

There is a strong body of evidence to suggest that different types of Ca channels regulate important functions, such as transmitter release, pacemaker activity, secretion and gene expression (Miller, 1987; Tsien et al. 1988).

Selective modulation of Ca channels by specific drugs is the technique most often used to assess the contribution of individual channel types to the overall function of

a cell. Such studies show that, in most cases, the biological function is rarely associated exclusively with an individual channel type.

The mechanism of transmitter release triggered by Ca entry into neurones has been studied by recording presynaptic Ca concentration with Ca-sensitive dyes and by controlling the levels of internal Ca with photosensitive Ca chelators. At fast synapses, it is suggested that $[Ca]_i$ decays slowly (in seconds) after presynaptic action potentials, whereas transmitter release lasts for only a few milliseconds after each spike. Simulations of Ca diffusing from Ca channels opened during action potentials suggest that neurotransmitter is released by a brief, localized increase in $[Ca]_i$ reaching about 100 μM (Augustine, 1987; Zucker, 1993b).

N-type current appears to represent a major component of voltage-dependent Ca current in neuronal cells. There is a strong evidence showing that the release of catecholamines in nerve terminals and sympathetic neurones is predominantly controlled by voltage-dependent Ca entry through N-type channels (Tsunoo et al. 1985; Hirning et al. 1988a; Lipscombe et al. 1988b). The depolarisation-induced secretion of the neurotransmitter is not, or only partially, reduced by organic blockers of L-type channels. 5-HT release in the hippocampus during electrical stimulation of the dorsal raphé (DR) area is blocked by cadmium, but not by dihydropyridine antagonists (Sharp et al. 1990). Other laboratories have pointed to a possible involvement of L-type channels during neurotransmission. Pharmacological studies of high potassium-induced transmitter release from cultured neurones (Hirning et al. 1988a) implicate N-type channels, e.g. norepinephrine release from sympathetic cultures or L-type channels, e.g. substance P release from DRG cultures, role in a transmitter release. Furthermore, ω-CgTx, a blocker of N-type channels, inhibited transmission at the frog neuromuscular junction but not at the same site in the mouse (Anderson & Harvey, 1987). The release of a putative neurotransmitter substance P from embryonic chick dorsal root ganglia and rat sensory neurones is inhibited by nifedipine, a blocker of L-type channels, in a voltage-dependent manner (Perney et

al. 1986; Rane et al. 1987). Catecholamine release from undifferentiated PC12 cells can be almost completely abolished by dihydropyridines (García et al. 1984; Ogura & Takahashi, 1984). However, dihydropyridines do not appear to affect substance P release from slices of adult rat spinal cord (Miller, 1987).

Furthermore, Wheeler et al. (1994) reported that, together with N-type channels, a novel Q-type channels might have a role in glutamatergic synaptic transmission at hippocampal CA3 - CA1 synapses. These results support the idea that neurotransmitter release may depend on multiple types of Ca channels under physiological conditions (Wheeler et al. 1994). Hirning et al. (1988a) proposed that Ca entry via N-type channels may dominate the release of small vesicles that contain only "classical" transmitters (such as catecholamines), whereas Ca influx through L-type channels can release larger vesicles containing peptides (such as substance P). The kinetic properties of the two channel types differ, so regular action potentials could allow an activation of N-type channels and promote a release of small vesicles, while intermittent bursts of impulses of high frequency might favour an activation of L-type channels and exocytosis from larger vesicles.

A role of DHP-sensitive L-type channels in contraction of cardiac and smooth muscle has been shown (Godfraind et al. 1986; Janis et al. 1987; Hosey & Lazdunski, 1988; Pelzer et al. 1990; Porzig, 1990). In many cases, Ca entry via L-type channels is required for hormone secretion in endocrine cells. Artalejo et al. (1994) examined the effect of Ca channels on secretion from chromaffin cells. Ca influx through the three types of Ca channels (N-, P- and L-type) triggered secretion individually, but P- and N-types were far less efficient, compared to L-type. In rat anterior pituitary cells, that possess both L- and T-type channels, depolarisation-induced Ca influx and hormone secretion was blocked with nanomolar concentrations of nimodipine, nisoldipine and nifedipine (Enyeart et al. 1985) and stimulated by the L-type channel activator BayK 8644 (Enyeart et al. 1985). However, these results certainly do not prove an exclusive role for DHP-sensitive channels in stimulus-secretion coupling.

It has been reported that synaptic stimulation is able to rapidly activate several transcription factor genes, such as *c-fos*, that are believed to be involved in modulation of gene expression that underlie neuronal plasticity (Sheng & Greenberg, 1990). In rat cerebral cortex, the expression of several transcription factor genes, such as *c-fos*, *fos-b* and *jun-B*, is suppressed in medium containing DHP antagonists, whereas BayK 8644 leads to an increased expression (Murphy et al. 1991). It is possible that a target for the transcription factor genes might be the expression of L-type channels. Further work is needed to clarify this interesting hypothesis.

T-type Ca channels are associated with current activation at relatively negative membrane potential and rapid inactivation (Fox et al. 1987b). This activation with relatively weak depolarisations has led to the suggestion that T-type current may be involved in pacemaker activity in spontaneously active neurones (Llinás & Yarom, 1981; Burlhis & Aghajanian, 1987). Hagiwara et al. (1988) showed that T-type current contributes importantly to the generation of pacemaker activity in spontaneously active neurones, such as in rabbit sinoatrial node. In line with this observation, it is possible that the bursting behaviour of various neurones in the mammalian CNS involves regenerative Ca current passing through T-channels (Burlhis & Aghajanian, 1987; Miller, 1987; Tsien et al. 1988; Bean, 1989b). Furthermore, in some cells, such as adrenal glomerulosa cells (Cohen & McCarthy, 1987), fibroblasts and smooth muscle cells (Chen et al. 1988), T-type channels appear to constitute a major voltage-gated Ca influx pathway and, therefore, might be essential for contractile and secretory events in these cells.

Effect of neurotransmitters on calcium currents

There are many observations that support the hypothesis that neurotransmitters modulate Ca channel currents (Dunlap & Fischbach, 1978; Reuter, 1983; Lipscombe & Tsien, 1987; Madison et al. 1987; Bean, 1989a; Dolphin et al. 1989; Penington et al. 1991; Elmslie, 1992; Menon-Johansson et al. 1993). This is a very substantial finding, because it means that neurotransmitters can alter many functions of Ca channels, and in this way affect plasticity of the transmission of signals in neuronal and non-neuronal cells.

These studies indicated that neurotransmitters can affect Ca channel currents in many mammalian central and peripheral neurones. At least two typical effects of neurotransmitters on Ca current have been reported in the majority of neurones. First, neurotransmitters can produce a change in the size of Ca current amplitude and, second, they can alter Ca current kinetics.

A fundamental question that had to be answered was whether a neurotransmitter modulates Ca channel current in the same way as Cd^{2+}. Cd^{2+} directly blocks Ca channels from the outside of the pore, whereas it was suggested that agonists reduce Ca current by acting through one or more transducing molecules (Catterall, 1993; Yang et al. 1993). If that is the case, the onset of the action of an agonist might be expected to be slower than that for Cd^{2+}. Surprenant et al. (1990) tested this hypothesis in guinea-pig submucosal neurones by comparing the latency of the action of NE and Cd^{2+}, when both were applied transiently with a pressure pipette. The group showed that a decrease in the Ca current amplitude was first observed with the depolarising pulse given 15 ms after the start of the Cd^{2+} ejection. In the presence of NE, a decreased current amplitude was detected with the stimulus given 220 ms after the first NE pulse. These observations suggested that Cd^{2+} and NE used different pathways to modulate Ca channel current.

Moreover, in sympathetic neurones NE probably selectively inhibits N-type Ca current rather than causing an overall reduction of Ca influx (Cox & Dunlap, 1992). Kongsamut et al. (1989) showed that the inhibitory effect of NE on Ca current is different from the effect of Cd^{2+}. NE eliminated a decaying component and partially inhibited a sustained component of Ca current in a voltage-dependent fashion. The inactivation time of NE-sensitive component was consistent with the kinetic properties of N-type channels. In contrast, Cd^{2+} inhibited Ca current without affecting the time course of the current decay. The same result was reported by Akasu et al. (1990) in rabbit vesical parasympathetic ganglion neurones.

Many studies suggested that a number of neurotransmitters modulate Ca current, see Table 1.1. In vertebrate neurones Dunlap and Fischbach (1978) showed that a number of transmitters, such as norepinephrine, GABA, 5-hydroxytryptamine, dopamine, enkephalin and somatostatin, but not acetylcholine or glycine, reduced Ca current in chick DRG neurones. Some of these actions of transmitters have been confirmed, e.g. NE's action on α-adrenergic receptors in rat superior cervical ganglion cells and enkephalin-induced inhibition of Ca current in mouse DRG cells (Werz & Macdonald, 1985). A common finding is that this inhibition is incomplete, and is usually between 30-60%, even at supramaximal concentrations of the neurotransmitters (10-100 μM), (Lipscombe & Tsien, 1987; Dolphin et al. 1989; Penington et al. 1991). The incompleteness of this effect raises questions concerning which type of Ca current is affected following transmitter application and whether different transmitters modulate different channel types.

In sympathetic ganglion neurones only 5-15% of Ca current is contributed by dihydropyridine sensitive L-type current (Boland & Bean, 1993). In the presence of 3μM nimodipine, 3 μM ω-CgTx blocked 97% of the remaining current. In this study, LHRH inhibited ω-CgTx sensitive but not nimodipine sensitive current, and, furthermore, had no effect on BayK 8644-enhanced Ca current. Therefore, it appears that LHRH inhibited N-type, but not L-type current. LHRH also had no effect on the

slowing of the tail current induced by BayK 8644 application, whereas it inhibited a fast component of the tail current. A similar conclusion was reached by Caulfield et al. (1992), when they examined the action of NE and acetylcholine on Ca current in neuroblastoma x glioma hybrid NG108-15 cells. NG108-15 cells contain three types (N-, L- and T-type) of Ca channels. Both ligands blocked Ca currents and the effect was not occluded either by an application of nifedipine, an L-type channel blocker, or Ni^{2+}, which is more selective for T-type channels. More conclusively, in the presence of ω-CgTx the modulation of Ca current was completely abolished.

Carbone and Lux (1987) examined the modulation of dopamine on Ca currents, in rat DRG neurones. Dopamine drastically inhibited Ca current. Similar effects of dopamine were observed in chick sympathetic neurones (Marchetti et al. 1986) and in clonal AtT-20 pituitary cell line (Brown et al. 1984). Both of these cell types lack T-type channels and AtT-20 cells possess only L-type channels. This latter result suggested that dopamine can act on L-type channels, rather than on N-type channels. This view is strengthened by the observation that the effect of dopamine was insensitive to changes in holding potential. Modulation of N- and L-type Ca channels by oxotremorine acting on muscarinic receptors was studied together with NE acting on α-adrenergic receptors in superior cervical ganglion neurones (Mathie et al. 1992). It was shown that NE inhibited only N-type current, whereas oxotremorine additionally depressed a DHP-potentiated tail current. In single-channel recordings, oxotremorine reduced the probability of opening of single N- and L-type channels, and acted via a diffusible second messenger. It appears that the compound activated two different pathways, one a diffusible second messenger signalling pathway inhibited both N- and L-type Ca channels, whereas a pathway activated by both oxotremorine and NE inhibited only N-type Ca current. This modulation of L-type Ca channels by oxotremorine was observed with 0.1 mM BAPTA, a potent chelator of intracellular Ca, in internal solution, whereas in the presence of 20 mM BAPTA the effect was abolished. With the higher level of BAPTA, the effect on N-type current was seen, with both NE and oxotremorine. This mechanism of action may

also be involved in the action of TRH mediated inhibition of HVA current in GH_3 cells, and kainate in hippocampal neurones (Nistri & Cherubini, 1991).

There have been reports of transmitters decreasing T-type current (Marchetti et al. 1986; Carbone & Lux, 1987; Schroeder et al. 1990; Schroeder et al. 1991). This may involve the activation of protein kinase C, since when activated by phorbol esters, this kinase was observed to selectively inhibit T-type current in rat DRG cells (Schroeder et al. 1990). T-type current has also been observed to be increased by neurotransmitters in spinal cord neurones (Berger & Takahashi, 1990) and hippocampal interneurones (Fraser & MacVicar, 1991).

Golard and Siegelbaum (1993) applied both NE and somatostatin simultaneously to chick sympathetic neurones to test whether the Ca current inhibition was additive. Initial application of somatostatin alone inhibited Ca current by 51%, whereas NE inhibited the current by 31%. When both compounds were applied together, the Ca current was inhibited by 41%, which was less than the inhibition seen with somatostatin alone. Thus, the inhibition was not additive, suggesting that the drugs acted possibly on the same population of Ca channels. It is possible that the two ligands activated the same population of inhibitory molecules that mediated the effect. The inhibitory molecule might be an activated G-protein. If this is so, NE and somatostatin may interacted with the same population of G-proteins. Alternatively, the compounds activated separate G-proteins, which then interacted with a common effector - a proposed inhibitory molecule. From the observations by Taussig et al. (1992) in hybrid rat-mouse NG108-15 cells, one could argue that these transmitters interacted with distinct G-proteins to inhibit Ca current. Indeed, in rat pituitary GH_3 tumour cell line somatostatin and muscarine have been shown to modulate DHP sensitive Ca current through two distinct subtypes of PTX sensitive G_o-protein (Kleuss et al. 1991).

As described, it is clear that a single application of many neurotransmitters

modulates Ca current. However, subsequent applications of the ligands desensitise Ca current (Huganir & Greengard, 1990; Elmslie et al. 1992). Desensitisation appears to be persistent, since the responses does not fully recover after washout of the drugs. Moreover, rather surprisingly the desensitisation is homologous and does not affect Ca current inhibition induced by other agonists (Elmslie, 1992). Bley and Tsien (1988) showed that the LHRH-induced inhibition of Ca current in a representative sympathetic neurone was subsequently weaker, following regular applications of the drug over a period of time. Interestingly, the response desensitised only when the neurones were examined using a perforated patch technique, but not in the whole-cell configuration. In perforated patch studies the cells are not dialysed with artificial pipette solution and the intracellular milieu is mostly preserved, whereas in the whole-cell studies, the pipette solution perfuses into the cell. Therefore, a mechanism of desensitisation involving an intrinsic "desensitisation factor" has been proposed (Ikeda & Schofield, 1989). However, there is still a disagreement whether a "desensitisation factor" exists in cells or not. Clearly, more studies are needed to understand this mechanism.

Modulation of voltage-activated Ca currents by different neurotransmitters appears to be voltage-dependent. Stronger block of Ca currents is observed at negative potentials, and it is largely removed at more positive membrane potentials (Marchetti et al. 1986; Bean, 1989a) or by a conditioning, strong depolarisation (Elmslie et al. 1990; Penington et al. 1991; Boland & Bean, 1993; Elmslie et al. 1993; Golard & Siegelbaum, 1993). Boland and Bean (1993) suggested that LHRH inhibited Ca current at all tested potentials, but the inhibition was much greater for small to moderate depolarisations than for large depolarisations. The group showed that a peak Ca inward current elicited by steps from V_H -90 mV to the test potential of -10 mV was inhibited by 28%, whereas Ca current evoked at the test potential of +130 mV was inhibited by only 11%. LHRH had no effect on the reversal potential. Voltage-dependency of LHRH-induced inhibition of N-type current could be analysed in more

detail using tail currents to assay the extent of the channel activation during test pulses to different voltages. In control experiments, using test pulses of 15-30 ms duration to activate Ca currents, the tail current was best fit by a single Boltzmann function. However, in the presence of LHRH, the tail current following small or moderate depolarisations decreased more than the one following larger depolarisations. The fit by a single Boltzmann function was inadequate in the presence of the transmitter and the best fit was a sum of two functions. Bean (1989a) gave additional evidence to support this theory by showing that in frog sensory neurones NE inhibited 54% of Ca current following small and moderate depolarisations, compared to 15% inhibition following test pulses to +130 mV. However, other reports contradict this view. Dolphin and Scott (1987; 1989) described an equal potency of inhibition of Ca current by baclofen following moderate and large depolarisations in rat sensory neurones. To explain this discrepancy, Beech et al. (1992) suggested that there may be at least two different fast pathways of modulation, one that is responsible for voltage-dependent and another one for voltage-independent modulation.

Many neurotransmitters produced a substantial slowing of the activation kinetics (Dunlap & Fischbach, 1978; Lipscombe & Tsien, 1987; Ikeda & Schofield, 1989; Jones et al. 1992; Golard & Siegelbaum, 1993). For example, Bean (1989a) showed that NE reduced Ca current amplitude and slowed the activation kinetics of the HVA channels in frog DRG cells. In the presence of NE, the activation curve was biphasic. However, an unexpected observation was made by Elmslie (1992), that in frog sympathetic neurones, the application of a variety of transmitters (LHRH, NE, muscarine, substance P) had no effect on the activation kinetics. The time constant of activation was identical to that in control experiments, i.e. in the absence of the drugs (Elmslie, 1992). This is in contrast with the results reported by Boland and Bean (1993) in the same cells. On the other hand, other groups have also observed the same effect as Elmslie on peripheral (Dunlap & Fischbach, 1981; Forscher et al.

1986; Ewald et al. 1988b), and central (Toselli et al. 1989; Sah, 1990) neurones.

Neurotransmitter-induced slowing of the activation kinetics of Ca current can be substantially relieved by large depolarising prepulses, applied just prior to the test pulses. This has been reported in many systems, such as in chick, rat and frog sympathetic neurones (Ikeda & Schofield, 1989; Boland & Bean, 1993; Elmslie et al. 1993; Golard & Siegelbaum, 1993), DRG neurones (Macdonald et al. 1986; Tatebayashi & Ogata, 1992), spinal cord neurones (Sah, 1990), dorsal raphé neurones (Penington et al. 1991), chromaffin cells (Artalejo et al. 1992a; Gandía et al. 1993) and NG108-15 cell line (Kasai, 1992).

With an addition of a prepulse the activation kinetics was usually completely restored, but the inhibition was never 100% relieved. Transmitter-induced inhibition of Ca current is generally best fit with a double exponential. The recovery from the block following the prepulse application was best fitted by a single exponential, as in control experiments. Similar changes in the activation kinetics are observed in different neuronal types (Ikeda & Schofield, 1989; Sah, 1990; Artalejo et al. 1992a; Boland & Bean, 1993; Elmslie et al. 1993). In a few cases longer prepulses fully restored the amplitude of Ca current to the control value (Kasai & Aosaki, 1989; Kasai, 1992).

Gray and Johnston (1987), among others (Takahashi & Berger, 1990), recorded not a reduction, but a potentiation of Ca current in the presence of a neurotransmitter. The group have shown that NE and the β-adrenoreceptor agonist isoprenaline, increased voltage-dependent Ca currents by 37%, in acutely dissociated granule cells from guinea-pig hippocampus. In this experiment, single-channel recordings demonstrated an enhancement of an inactivating 14 pS channel by these agonists. The fact that this effect of NE is mediated by β-adrenoreceptors is in contrast to the reduction of N-type current which seems to be α_2-adrenoreceptor related (McFadzean et al. 1989). The described action of NE and isoprenaline could be mimicked by a

forskolin-induced activation of adenylyl cyclase or by a direct application of a membrane-permeable analogue of cAMP. The potentiation of Ca current may be analogous to the β-adrenoreceptor-induced increase in L-type current observed in heart cells, that probably involves cAMP-dependent phosphorylation of L-type channels (Bean, 1985; Hagiwara et al. 1988; Hartzell et al. 1991). This finding suggests that an intracellular second messenger might have a role in modulation of Ca channels.

An involvement of G-proteins in the transmitter-induced modulation of Ca currents has been strongly indicated, see *Chapter 1.6.* Most neurotransmitters reported to inhibit HVA Ca current are thought to require an activation of GTP-binding proteins (Holz et al. 1986; Dolphin & Scott, 1989; Porzig, 1990; Cox & Dunlap, 1992; Campbell et al. 1993; Dolphin et al. 1993), for review see Dolphin (1990; 1991a; 1995). The slow turn-on of Ca current in the presence of neurotransmitters might merely reflect a time- and voltage-dependent uncoupling of G-proteins from HVA channels at depolarised membrane potentials. Thus, whether slowing of Ca channel activation and Ca current depression reflects a singular mode of action or two independent actions on two pharmacologically distinct Ca channels remains to be seen.

One idea, supported by Dunlap (Dunlap & Fischbach, 1978; Cox & Dunlap, 1992) is that the slowing of HVA current activation kinetics is associated with some, but not all, G-proteins, and that differences in transmitter effects on the current kinetics reflect the selectivity of receptor-G-protein interactions. Dunlap and Fischbach (1978) argued that NE reduced the number of functional calcium channels in the cells. In contrast with that hypothesis, Bean (1989a), has suggested that the inhibition of Ca currents is primarily due to a transmitter-induced change in the voltage-dependency by which the channels are opened. There is little or no change in the number of functional channels activated by very large depolarisations. His argument is that in

the control cells, i.e. in the absence of neurotransmitters, at any time the majority of the channels are in the "willing" mode to open and that a neurotransmitter increases the fraction of channels in the "reluctant" mode.

Role of G-proteins and second messengers

Neurotransmitters bind to membrane receptors that can be classified into two major groups. These two receptor groups differ in transmembrane topology, oligomeric structure and in the molecular mechanisms by which they transduce signals, for review see Huganir and Greengard (1990). One group of the membrane receptors includes $GABA_A$, nicotinic acetylcholine, NMDA, glycine and kainate receptors. These receptors contain a receptor binding site, as well as an ion channel that transduces signals, and all but NMDA receptor possess fast activation kinetics. The second group of receptors are associated with GTP-binding (G) proteins, which possess slower kinetics and consist of α-adrenergic, β-adrenergic, dopamine, muscarinic acetylcholine, some serotonin and many neuropeptide receptors, for reviews see Reuter (1983), Gilman (1987), Dolphin (1990; 1991a; 1995), Hess (1990), Pelzer et al. (1990), Taylor (1990) and Clapham (1994).

There is a strong evidence for the presence of G-protein-coupled receptors that modulate Ca currents. For example, cardiac L-type Ca channels, appear to be modulated by G_s and G_i, via their control of adenylyl cyclase activity (Cachelin et al. 1983; Hescheler et al. 1987). There are also suggestions that G_s could interact directly with L-type Ca channel to increase its open state probability (Yatani et al. 1988). Andrade (1993) showed that an activation of G_i by 5-HT or $GABA_B$ receptor stimulation enhanced the ability of G_s to stimulate adenylyl cyclase(s) in rat

hippocampal neurones.

Signal transducing G-proteins consist of α-, β- and γ-subunits. The diversity among the three better characterised G-protein subtypes, G_s, G_i and G_o, is basically dependent on different α-subunits. This subunit probably represents an active part of G-proteins, whereas a role of βγ-complex is less clear.

Direct confirmation for the involvement of G-proteins in Ca current modulation comes from the experiments where neuronal cells were treated with pertussis toxin (PTX), cholera toxin (CTX), antisense oligonucleotides and GTP-γ-S. PTX prevents receptor-mediated effects by interaction on mainly G_o and G_i, while CTX opposes GTP hydrolysis by ribosylating G_s. It has been reported (Hescheler et al. 1987; McFadzean et al. 1989; Menon-Johansson et al. 1993) that the α-subunit of G_o, $G\alpha_o$, is involved in transducing signals from a number of different receptors to Ca channels in neurones and secretory cells, and other researchers have shown $G\alpha_i$ also to be effective (Surprenant et al. 1990). Therefore, if the action of neurotransmitters is regulated by G_o, in the presence of PTX the effect will be abolished. Indeed, in the presence of the toxin, the opiate agonist DADLE had no effect on Ca current in NG108-15 cells. However, intracellularly injected G_o, and to a lesser degree G_i, restored the inhibitory effect of this agonist on Ca current. Since G_o was ten-fold more effective, it was suggested that it is probably G_o that couples δ-opioid receptors to Ca channels (Hescheler et al. 1987). Ewald et al. (1988b) reported a similar finding. They examined the effect of neuropeptide Y (NPY) in DRG cells, and proposed that G_o mediated the inhibitory effect of NPY on Ca current. This effect on Ca channels appears to be voltage-dependent; the block was stronger during hyperpolarisation and decreased with larger depolarisations (Tsunoo et al. 1986). However, PTX does not prevent the action of bradykinin on Ca current in neuroblastoma cells (Taussig et al. 1992) and its differential actions are also shown in rat superior cervical ganglion cells. In these neurones the action of acetylcholine was completely abolished, but NE-induced inhibition was only partially reduced

(Song et al. 1989).

G_s, a G-protein that supposedly stimulates adenylyl cyclase, potentiated the stimulatory effect of BayK 8644 on Ca current in myocardium (Yatani et al. 1987; Yatani et al. 1988). This effect was abolished in the presence of CTX. Furthermore, the opening probability of Ca channels from skeletal muscle *t*-tubules, reconstituted into lipid bilayers, was enhanced by activated G_s protein or by its α-subunit in the presence of BayK 8644 (Yatani et al. 1988). Second messengers, such as cAMP, appeared not to be involved in this modulation. Therefore, it appears that G-proteins help to maintain the Ca channel protein in an "activatable" state, possibly by stabilizing the resting or open state of the channel. However, if this is true, G-proteins should be expected to antagonise the effects of Ca channel blockers which act by stabilizing the inactivated state of the channels. Such a loss of blocking potency has, indeed, been observed. L-type channel blockers verapamil and d-cis-diltiazem had no inhibitory effect on Ca current in the presence of internal GTP-γ-S, in dorsal root ganglion neurones (Scott & Dolphin, 1987).

GDP analogues inhibit G-proteins and oppose the response to neurotransmitters. It was an interesting observation that their inclusion increased the peak amplitude of Ca current in DRG neurones, with a specific effect on the transient component of the current (Scott & Dolphin, 1986; Dolphin & Scott, 1987). This led to a suggestion that under basal conditions G-proteins maintain a fine balance between activatable and inactive state.

Another way to identify G-proteins involved in modulatory effects on Ca current is to use antisense oligonucleotides to abolish expression of G-protein α-subunits and then test the action of an agonist. By intranuclear injection of antisense oligonucleotides that have complementary sequences to $G\alpha_o$ and $G\alpha_i$, into rat pituitary GH_3 cells, Kleuss et al. (1991) suggested that G_{o1} and G_{o2}, but not G_i, mediated Ca current inhibition following activation of muscarinic and somatostatin

receptors, respectively. Studies by Campbell et al. (1993) have identified G_o, but not G_i, to be responsible for $GABA_B$ receptor coupling to voltage-sensitive Ca channels in cultured rat DRG neurones. The effect was studied in a preparation where $GABA_B$ receptors are selectively coupled to N-type channels. However, it is too early to conclude that G_o couples to a specific type of Ca channels, and since there are no dihydropyridine sensitive channels in this cell line, it is unclear whether G_o can couple to neuronal L-type channels.

In many cell types, neurotransmitter-induced modulation of Ca currents following activation of G-proteins, can be mimicked by an intracellular administration of guanosine 5'-O-(3-thiotriphosphate), GTP-γ-S. GTP-γ-S is a non-hydrolysable GTP analogue that permanently activates G-proteins. GTP-γ-S, included in a recording pipette, reduced Ca current amplitude and caused a marked slowing of the activation kinetics (Lewis et al. 1986; Dolphin & Scott, 1987; Penington et al. 1991).

Furthermore, there has been some controversy whether G-proteins interact exclusively with one or more types of Ca channels. Researchers argued that by a stimulation of G-protein predominantly N-type current was inhibited, as discussed earlier. On the other hand, other groups showed that G-proteins also acted on L- and possibly T-type Ca current. For example, studies have suggested that the effect of L-type Ca channel activators, such as BayK 8644, was strongly enhanced following intracellular application of GTP-γ-S. The effect was abolished in the presence of PTX (Scott & Dolphin, 1988).

There have been fewer studies of the modulatory effect of neurotransmitters following G-protein activation on the LVA Ca current. It was described that dopamine and the adenosine agonist 2-chloroadenosine (2-CA) inhibited T-type current in chick and rat DRG neurones (Forscher et al. 1986; Scott & Dolphin, 1987). Scott et al. (1990) observed that a photorelease of 10-20 μM GTP-γ-S intracellularly from a photosensitive nitrophenylethyl ester of GTP-γ-S inhibited only T-type current

in rat DRG cells, whereas a lower concentration of GTP-γ-S, increased the current.

As described, there are examples where an activation of various G-proteins resulted in different actions on Ca channels, but it appears that, at least in some neuronal types, an activation of different G-proteins can induce identical changes in Ca channel gating. Elmslie (1992) reported that in bullfrog sympathetic neurones NE and LHRH inhibited Ca current. An application of LHRH occluded the response to NE, but, LHRH was still effective following application of NE. Furthermore, a PTX treatment of the cells or dialysis with the A-protomer of PTX, markedly reduced the effect of NE, but not that of LHRH on Ca channel current. This suggested that more than one G-protein was involved in modulation of the current, although both drugs showed similar effect, that was an inhibition of Ca current. It appears that the adrenergic receptor preferentially couples to the PTX sensitive G-protein, while the LHRH receptor can interact with a PTX insensitive G-protein(s). It is possible that different G-proteins modulate the same Ca channels, because each transmitter inhibited more than 50% of the current, and the effect of two neurotransmitters applied simultaneously was partially additive. In addition, Ca currents evoked were kinetically identical. Very similar observation was reported by Taussig et al. (1992). This group suggested that different G-proteins regulate modulation of N-type Ca current in NG108-15 cell line. These cells contained a PTX resistant mutant of G_{oA}. Leu-enkephalin, NE and somatostatin all inhibited Ca current and the effect was ω-CgTx sensitive. PTX blocked the action of somatostatin, but not that of leu-enkephalin or NE. Thus, while NE and leu-enkephalin's actions were probably mediated via G_{oA}, somatostatin acted via a different PTX sensitive G-protein. It appears that each G-protein is highly specific in the choice of the receptor and that different G-proteins can activate a common effector upon their activation.

Dolphin (1991a; 1991b) hypothesised that two mechanisms could be involved in the G-protein regulation of Ca channels. Upon activation, G_o or G_i interacted with the

Ca channels in their resting state, resulting in a voltage-dependent inhibition. The slow activation rate could then be explained as a function of the slow dissociation, or unbinding, of this complex and the subsequent opening of the channels upon depolarisation (Dolphin et al. 1989; Grassi & Lux, 1989). In favour of this model, it was shown that a slow activation could be speeded up by a brief depolarising prepulse. The second reason for the reduction of the amplitude of Ca current by neurotransmitters can be due to an involvement of a freely diffusible second messenger. Possibly, G_i activation and a consequent inhibition of adenylyl cyclase, resulted in a reduced cAMP-dependent Ca channel phosphorylation. It was shown that in heart and skeletal muscle Ca channels were possible substrates for cAMP-dependent phosphorylation on the α_1- and β-subunits (Takahashi et al. 1987; Hartzell et al. 1991; Andrade, 1993). This resulted in a potentiation of Ca currents and in an increase of the open probability and the number of functional Ca channels (Rosenberg et al. 1986; Fox et al. 1987a). However, the response to cAMP in neuronal Ca channels is less clear.

There is an evidence to suggest that at least in some cells a modulation of Ca current by neurotransmitters imply a direct interaction between G-proteins and various Ca channels. However, this appears not to be a general rule. Some groups have already proposed (Dolphin, 1990; Bernheim et al. 1991; Dolphin, 1991b; Braha et al. 1993; Sculptoreanu et al. 1993; Netzer et al. 1994; Rosenmund et al. 1994), that binding of a ligand to G-protein activates another, highly specific intracellular mechanisms. Bernheim et al. (1991) showed that muscarinic, but not α-adrenergic, action on Ca channels is mediated by a diffusible second messenger.

The potential mechanisms responsible for modulation of Ca current in the presence of neurotransmitters could be divided into four categories:

1. cAMP-dependent phosphorylation

2. activation of phospholipase C and PKC
3. other second messengers (e.g. arachidonic acid)
4. direct G-protein - Ca channel interaction

First, neurotransmitters could stimulate and/or inhibit adenylate cyclase, by activation of G_s or G_i, respectively. For example, norepinephrine increases Ca current and the effect results from the coupling of β-receptors with G_s, that triggers cAMP-dependent phosphorylation of L-type Ca channel (Cachelin et al. 1983; Kameyama et al. 1986; Sculptoreanu et al. 1993). A similar mechanism was described for a regulation of L-type channels in other cells (Armstrong & Eckert, 1987), but whether all L-type channels are sensitive to elevations of intracellular cAMP remains unclear. However, Hartzell et al. (1991) showed that in frog, rat and guinea-pig ventricular myocytes, an increase in Ca current was due to cAMP phosphorylation, without G-protein activation.

Chad and Eckert (1986) and other researchers (Hill & Dolphin, 1984; Dolphin, 1991b; Dolphin, 1992) also observed that other compounds, such as forskolin, increased cAMP levels and enhanced Ca current in rat DRG neurones. However, forskolin was not able to prevent the inhibitory effect of neurotransmitters (Akaike et al. 1989; Dolphin, 1991b; Frace & Hartzell, 1993).

Secondly, as a hypothesis, neurotransmitters could activate phospholipase C, possibly by a distinct G-protein (G_q). In most cases, a PTX insensitive mechanism is involved in such modulation. This evidence mostly relies upon the use of the PKC activators phorbol esters and diacylglycerols. Activation of PKC is thought to underlie the inhibition of Ca channels by NE in chick DRG neurones and NPY in rat DRG cells (Rane & Dunlap, 1986). In rat hippocampal cells PKC activation also inhibited Ca current (Doerner et al. 1988). Activation of PKC has been reported to increase (Dösemeci et al. 1988; Lacerda et al. 1988) or decrease (Lacerda et al. 1988) DHP sensitive Ca current in cardiac and smooth muscle cells. In contrast, there is a

strong evidence that inhibitory actions of many other putative transmitters do not appear to involve PKC, since PKC activation does not produce the characteristic voltage-dependent inhibition of Ca current (Wanke et al. 1987; Bley & Tsien, 1988; Akaike et al. 1989; Dolphin et al. 1989; Kasai & Aosaki, 1989; Plummer & Hess, 1991). Inhibitors of PKC, like H-7, a non-specific PKC and protein kinase A (PKA) inhibitor, polymixin B (PKC blocker) and staurosporine (a potent non-specific kinase inhibitor) were unable to prevent the reduction of Ca currents caused by neurotransmitters (Penington et al. 1991; Dolphin, 1992). Therefore, it is most likely that in these cells PKC activation is not necessary for the neurotransmitter-induced reduction of Ca current.

It might be possible that some other second messengers regulate modulation of Ca current; it was proposed that an eicosanoid, arachidonic acid, mediated the inhibition of Ca currents in *Aplysia* sensory neurones (Brezina et al. 1987; Schacher et al. 1993). The effect was regulated by a PTX sensitive G-protein. However, in other cells, as in DRG (Dolphin et al. 1989) and DR (Penington et al. 1991) neurones this action of arachidonic acid was not observed.

On the other hand, evidence is mounting to suggest that, at least in some cells, a coupling between transmitters and G-proteins can directly modulate Ca channels. Data in support of such a direct G-protein-ion channel modulation is based on several observations. First, a number of studies have failed to identify any second messenger that mimics the action of neurotransmitters. Second, transmitter-dependent inhibition of Ca current can be quite rapid, with an onset occurring in 50 ms, which is faster than most known second messenger-mediated events (Bean, 1989a). Third, application of a transmitter to the bath fails to inhibit Ca current in cell-attached patches, arguing against a freely diffusible second messenger and supporting a membrane-delimited mechanism of G-protein action (Forscher et al. 1986; Lipscombe et al. 1988b).

A direct interaction between G-proteins and Ca channels has already been proposed in cardiac myocytes (Yatani et al. 1987). With that in mind, Kasai and Aosaki (1989)

discussed the data obtained in chick sensory neurones. The adenosine analogue 2-CA selectively reduced ω-CgTx sensitive Ca channel current via G-protein activation. cAMP, cGMP, PKC or eicosanoids were unable to prevent the neurotransmitter effect, suggesting that the G-protein acted directly on the channel. A similar idea is supported by Kozlowski et al. (1994). They described a direct interaction of G_s with L-type Ca channels in cardiac myocytes.

Boland and Bean (1993) proposed a model that assumes that the inhibition by transmitters results from direct interaction between G-protein(s) and Ca channel. This model is based on two assumptions: a) that G-protein stabilizes closed gating states (see above) and b) activation of G-protein-bound channels destabilizes the binding of the G-protein to the channel. The model acknowledges the previous hypotheses (Bean, 1989a; Elmslie et al. 1990) that there might be two, "reluctant" and "willing", modes of gating of Ca channels and that conversions between the two modes represent binding and unbinding of G-proteins. In Bean's view, there is a possibility that the "reluctant" mode represents gating of channels modified by direct binding of activated G-proteins to Ca channel. This is in opposition to the model proposed by Kasai and Aosaki (1989) and Kasai (1992), which is based on a speculation that gating states of Ca channels reflect slow gating transitions in a channel which is continuously modified by G-protein binding.

Serotonin and calcium currents in dorsal raphé neurones

Serotonin (5-hydroxytryptamine, 5-HT) is a putative neurotransmitter, that was discovered by Rapport et al. in 1948. It has been shown that the compound plays a major role in multiple neurologic and non-neurologic states, such as pain, sleep, migraine, appetite, anxiety, depression and aggression (Sternbach, 1991).

The largest density of serotoninergic neurones is found in dorsal raphé (DR) nucleus. Other areas of the brain with an abundancy of 5-HT receptors are septum, hippocampus, entorhinal cortex, cerebral cortex, thalamic and hypothalamic nuclei and interpeduncular nucleus (Steinbusch, 1981; Bowker et al. 1983; Montone et al. 1988; Ma et al. 1991; Matsuzaki et al. 1993). The DR nucleus, located in the periaqueductal grey in the mesencephalon, sprouts a large amount of both ascending and descending fibres, for review see Steinbusch (1981). The ascending bundle divides and forms the dorsal and ventral serotoninergic pathways. These fibres terminate in basal ganglia and other centres in diencephalon and telencephalon (Matsuzaki et al. 1993). The descending bundle of DR nucleus ends in cerebellum, nucleus of the solitary tract, lower sections of medulla and spinal cord, such as spinal trigeminal nucleus and dorsal horn of spinal cord (Li et al. 1993).

There is a strong body of evidence to suggest that in the descending serotoninergic nerve terminals 5-HT co-exists with one or more other putative neurotransmitters, such as substance P, enkephalin, thyrotropin-releasing hormone, GABA and catecholamines. For example, immunohystochemical studies using specific antibodies to serotonin and the peptides, showed that a large majority of raphé neurones projecting to the spinal cord are serotoninergic and that about half of them contain substance P immunoreactivity. Only a few neurones contained 5-HT alone (Bowker et al. 1983). It is possible that an interaction in the release of these substances finely tunes important physiological processes, such as sleep and modulation of pain sensation. Moreover, these neurones with such widespread distribution at both higher and lower levels of the nervous system also control specific behavioural and autonomic activities, such as food intake, arousal, innervation of respiratory muscles and regulation of cardiac function, in association with other neuronal groups.

Approximately 90% of the neurones in DR region possess 5-HT_{1A} receptors (Verge et al. 1985; Lawrence et al. 1989). 5-HT_{1A} receptors, first cloned by Kobilka et al.

(1987), appear to be localized on the cell bodies and dendrites of DR neurones. Their role in autoinhibition of the 5-HT release has been proposed (Verge et al. 1985; Becquet et al. 1990; Wu et al. 1991). This hypothesis is supported by biochemical (Hjorth et al. 1982), lesion (Verge et al. 1985) and electrophysiological (Sprouse & Aghajanian, 1988; Penington & Kelly, 1990) studies. The 5-HT agonist 8-hydroxy-2-(di-n-propylamino)tetraline, 8-OH DPAT, binds with a high affinity (K_i of 1.45 nM) and specificity (K_D of 24 nM) to 5-HT_{1A} receptors (Verge et al. 1985). The binding of the drug decreased dramatically after deliberately induced degeneration of 5-HT cell bodies with 5,7-dihydroxytryptamine (Verge et al. 1985). This has been confirmed by demonstration of a close correlation between [^{3}H]8-OH DPAT binding and 5-HT_{1A} mRNA in situ hybridisation histochemistry in DR cells (Chalmers & Watson, 1991).

DR neurones exhibit pacemaker potentials (Aghajanian & VanderMaelen, 1982; Burlhis & Aghajanian, 1987; Penington et al. 1991). Electrophysiological studies demonstrated a slow, regular firing pattern which was maintained by addition of α_1-adrenoreceptor agonists (VanderMaelen & Aghajanian, 1983). 8-OH DPAT also affected the DR firing rate by increasing potassium conductance, mimicking the effect of 5-HT (Sprouse & Aghajanian, 1988; Hećimović et al. 1992). This increase in potassium conductance can be blocked by an application of (-)-propranolol, a 5-HT_{1A} receptor antagonist (Sprouse & Aghajanian, 1986). Intracellular current-clamp recordings of DR neurones in vivo (Aghajanian & VanderMaelen, 1982) as well as in vitro (Pan et al. 1989; Penington et al. 1992), unveiled a large afterhyperpolarisation after each action potential followed by a slow decay during the interspike interval. Burlhis and Aghajanian (1987) suggested that these spikes arise from depolarising ramps, rather than from excitatory postsynaptic potentials. Spontaneous action potentials in these cells measured 70-100 mV and they can also be evoked by depolarising steps positive to -60 mV. Application of tetrodotoxin (TTX) onto depolarised DR cells uncovered low- and high-threshold calcium-

dependent spikes. The low-threshold spike was inactivated by holding potentials positive to -50 mV (Penington et al. 1991). The membrane potential of DR neurones is in the range of -40 to -60 mV.

It was also described that 5-HT and the 5-HT agonist d-lysergic acid diethylamide (LSD) had an inhibitory effect on the repetitive firing of DR cells, possibly through a proposed "somatodendritic 5-HT_{1A} autoreceptor" (Aghajanian & VanderMaelen, 1982; VanderMaelen & Aghajanian, 1983). Both drugs hyperpolarised the neurones as shown in current-clamp recordings, due to an increase in an inwardly rectifying potassium conductance (Williams et al. 1988; Kelly et al. 1991; Pan et al. 1993). 8-OH DPAT has a similar depressant effect on DR firing rate as LSD and 5-HT, as does ipsapirone, another selective 5-HT_{1A} ligand (Sprouse & Aghajanian, 1986). The actions of both drugs have also been shown to increase a potassium conductance similar to that mediated by 5-HT.

In recent studies (Penington & Kelly, 1990; Penington et al. 1991) on acutely isolated adult rat DR neurones, intra- and extracellular solutions were made to isolate Ca current. At a holding potential of -100 mV a peak Ca current amplitude was obtained with a voltage step to -10 mV. At the end of a 150 ms long test pulse, the peak current was only partially inactivated. Most of the HVA Ca current is blocked by ω-CgTx and possessed properties typical for N-type current. Switching the holding potential from -100 mV to -50 mV, the peak Ca current had smaller amplitude, showed little or no inactivation and was partially DHP sensitive, having some of the properties of L-type current (Penington et al. 1991). Depolarisation jumps from V_H -80 mV to the test potentials of -50 to -35 mV, elicited a small inward current that rapidly inactivated, characteristic of T-type Ca current seen in dorsal root ganglion cells (Fox et al. 1987a; Fox et al. 1987b; Bean, 1989b; Schroeder et al. 1990).

The presence of different Ca current types in DR neurones was confirmed in

whole-cell and single-channel recordings. There is evidence to suggest an existence of at least four types of Ca current in DR neurones. Channel openings with a slope conductance of 8 pS with small depolarisations were consistent with T-type channels. Larger depolarisation steps elicited openings with larger conductance of 23 pS and the application of BayK 8644, a DHP agonist, prolonged the opening time. The third channel had a conductance of 15 pS and was ω-CgTx sensitive (Penington et al. 1991).

It appears that the peak current evoked from a holding potential of -100 mV to -10 mV has both N- and L-type components of Ca current. Studies with ω-CgTx and DHPs have been performed to examine this. However, an administration of 1 μM ω-CgTx blocked 40% of the peak Ca current and the addition of 1 μM nimodipine, a DHP antagonist, further reduced the current by only 4% (Penington et al. 1991). This result shows that a significant amount, ~50%, of the HVA current was not of N- or L-type. It is, therefore, possible to conclude that DR neurones possess N-type current, a small and variable L- and T-type components and a significant amount of Ca current that is ω-CgTx and DHP insensitive.

As described, in many neurones it has been shown that neurotransmitters modulate Ca current (Sah, 1990; Fraser & MacVicar, 1991; Elmslie, 1992; Gandía et al. 1993). However, the exact mechanism is still unclear. 5-HT and 8-OH DPAT inhibited the peak Ca current in DR neurones by 50% and 27%, respectively, and it appears that HVA, but not LVA current is affected by these compounds (Penington & Kelly, 1990). The drugs also produced a dramatic slowing of the activation of the current. The effect was blocked by the 5-HT_{1A} antagonist 1-(2-metho-oxyphenyl)-4-[4-(2-phthalimmidobutyl]piperazine (NAN 190), suggesting this action was mediated by the 5-HT_{1A} receptor subtype (Glennon et al. 1988; Rydelek-Fitzgerald et al. 1990). It, therefore, appears that the same autoreceptor causes both hyperpolarisation via an increase in potassium conductance, and inhibits HVA Ca current, and both of these actions have been shown to occur simultaneously in the same cell (Penington et al.

1992). A similar observation was reported by Koike et al. (1994) who described that 8-OH DPAT reversibly inhibited N-type current in acutely isolated ventromedial hypothalamic neurones. Ciranna et al. (1993) used porcine pituitary intermediate lobe cells and showed an inhibition of Ca current by 5-HT and to a smaller degree by 8-OH DPAT. They suggested that the effect was mediated via G-protein and proposed a direct interaction between the G-protein and Ca channels, because no diffusible second messenger was identified. On the other hand, Berger and Takahashi (1990) unexpectedly showed that 5-HT increased Ca current and acted mainly on LVA channels in neonatal rat spinal motoneurones.

It is still too early to conclude which type of Ca current is inhibited by an application of the 5-HT_{1A} agonists in DR neurones. In the presence of ω-CgTx the inhibition of the peak Ca current amplitude was reduced from 50% to 40% (Penington et al. 1991). The percentage of the inhibition was not substantially affected by the change in holding potential from -100 mV to -50 mV. It appears that both ω-CgTx sensitive and insensitive Ca currents were modulated. Complexity of the modulatory action of neurotransmitters on Ca current has also been described in other neuronal types.

The inhibition of the peak Ca current by 8-OH DPAT in DR neurones is sensitive to a prepulse application. A brief and strong depolarising prepulse to 40 mV, applied just prior to the test pulse, partially relieved this inhibition (Penington et al. 1991). Such pulses have little or no effect on control currents.

Various reports suggested that the action of agonists on 5-HT_{1A} receptor is mediated by G-protein, probably G_o or G_i subtype (Innis et al. 1988; Penington et al. 1991). For example, intraventricular injection of pertussis toxin eliminated the 5-HT evoked hyperpolarisation in hippocampus (Andrade & Nicoll, 1987). GDP-β-S abolished the 5-HT induced hyperpolarisation in DR neurones. Moreover, GTP-γ-S mimicked the action of 5-HT and produced a sustained hyperpolarisation (Andrade

& Nicoll, 1987; Innis et al. 1988). Intracellular perfusion of DR neurones by GTP-γ-S had the same effect as 5-HT and reduced the peak amplitude of Ca currents (Penington et al. 1991; McAllister-Williams, 1992). Therefore, it appears that the 5-HT action is G-protein mediated. Electrophysiological studies were unable to give strong evidence that changes in $[Ca^{2+}]_i$ and in cAMP levels mediate the 5-HT action on Ca currents. H-7, phorbol esters, 8-Br-cAMP and arachidonic acid were ineffective in preventing the inhibitory effect, suggesting that protein kinases are probably not involved (Penington et al. 1991). Furthermore, 5-HT applied externally, in cell-attached single-channel recordings, had no effect. It appears that the inhibition of the peak Ca channel currents by 5-HT in DR neurones is mediated via 5-HT_{1A} receptor coupled to a PTX-sensitive G-protein, without the involvement of a freely diffusible intracellular messenger.

The inhibitory effect on the peak Ca current and slowing of the activation kinetics following activation of 5-HT_{1A} receptor in DR neurones, are similar to the action of various other neurotransmitters on different cells (Kasai & Aosaki, 1989; Boland & Bean, 1993). It is physiologically significant that 5-HT modulates Ca channel currents. Release of 5-HT from the nerve terminals of DR neurones is under autoinhibitory control (Becquet et al. 1990; Wu et al. 1991). Thus, the entry of Ca ions into raphé terminals could trigger a release of 5-HT and other substances and, with a negative-feedback mechanism following an activation of somatodendritic 5-HT_{1A} receptors, 5-HT might regulate this release. For example, 5-HT applied to the DR causes a rapid inhibition of the release of 5-HT from axonal nerve endings at sites distant to the DR (Becquet et al. 1990). This is highly significant, since 5-HT can be synthesized and released in the DR nucleus itself. 5-HT-mediated hyperpolarisation decreases DR firing rates, and possibly reduces both local and distal release of 5-HT. In some other neuronal types it has already been proposed that 5-HT could inhibit transmitter release by acting on 5-HT_{1A} receptors (Sharp et al. 1993) or by an inhibition of synaptic potentials (Bobker & Williams, 1990).

Protein phosphatase inhibition

Inhibition of dephosphorylation in different cell types has been shown to modulate Ca currents (Hescheler et al. 1988; Artalejo et al. 1990; Dolphin, 1992; Berlin & Preston, 1993; Braha et al. 1993; Elmslie et al. 1993; Frace & Hartzell, 1993; Ono & Fozzard, 1993; Werz et al. 1993; Betz & Henkel, 1994). Thus, clearly, an investigation of this dependence of Ca channels on phosphorylation could aid a better understanding of the mechanism involved in the regulation and expression of Ca currents in various cell types.

Protein phosphatase 1 and 2A inhibitors

Okadaic acid (OA), microcystin-LR (MC) and calyculin-A are shown in biochemical experiments to inhibit specifically protein phosphatase 1 (PP1) and 2A (PP2A), with different potencies (Ingebritsen & Cohen, 1983; Nishiwaki et al. 1990). These analogues belong to the OA group of compounds, but have structural and, to a certain extent, functional diversities. For that reason, they will be introduced separately.

Okadaic acid

Okadaic acid is a polyether compound isolated from a black sponge *Halichondria okadai* (Bialojan & Takai, 1988). The drug binds to protein phosphatase 1 and 2A, and inhibits their activity. This inhibition leads to a prolonged phosphorylation of proteins in the cells (Nishiwaki et al. 1990; Fujiki & Suganuma, 1993). It appears that the carboxyl group, as well as the four hydroxyl groups at C-2, C-7, C-24 and C-27 of OA, are important for its function. It has also been evaluated as possible

tumour promoter, for review see Fujiki and Suganuma (1993).

Chemical structure of OA was determined by Tachibana et al. (1981) and Bialojan and Takai (1988) reported that OA acted as a non-competitive or mixed inhibitor on the OA-sensitive protein phosphatases. Therefore, the binding site for OA might be different from that for the substrate. OA probably interacts directly with a catalytic subunit of the protein phosphatases (Nishiwaki et al. 1990).

Furthermore, the drug is ~50-100 times more potent in blocking PP2A than PP1 (Haystead et al. 1989; Fujiki & Suganuma, 1993). The order of potency for other related compounds for the inhibition of PP2A is: OA > MC > calyculin-A. The reverse is true for the inhibition of PP1; MC and calyculin-A appear to be the most potent. The proposed order of potency is: MC > calyculin-A > OA.

So far, there has been no strong evidence given to explain this difference in the potency of inhibition of PP1 and 2A. One speculation is that the effect reflects different catalytic subunits of the two phosphatases. However, in the opposition to this theory is the observation that the two phosphatases have a striking sequence homology between their catalytic subunits. Another possibility is that the non-catalytic subunits make the catalytic subunit less accessible to OA (Nishiwaki et al. 1990). It was described earlier that the OA site and the substrate site are different, and, possibly, the affinity of OA for the phosphatase is considerably decreased in the presence of a substrate (Nishiwaki et al. 1990).

When OA inhibits the protein phosphatases that presumably leads to an activation of serine/threonine group of protein kinases (Sassa et al. 1989). It appears that the action of protein phosphatase 1 and 2A on the phosphorylate kinases is slightly different. It is postulated that PP1 dephosphorylates β-subunit of a phosphorylase kinase, whereas PP2A preferentially dephosphorylates the α-subunit (Ingebritsen & Cohen, 1983).

The exact protein kinase(s) that mediate this process are yet to be identified. Frace

and Hartzell (1993) were unable to observe an involvement of protein kinase A, protein kinase C, tyrosine kinases and calcium-calmodulin kinase II in the modulation of Ca currents in cardiac myocytes in the presence of OA. They suggested that OA and the tested kinases all activated different biochemical pathways.

The present understanding of the OA pathway is still very hypothetical. There is an evidence to suggest that OA binds to PP1 and PP2A in the cell membrane and is incorporated into the cells. Intracellularly, OA inhibits activities in the cytosol and nucleus, which leads to an increase in phosphorylation of proteins, such as hyperphosphorylation of intermediate filaments and possibly regulatory proteins of different membrane channels. Furthermore, in the nucleus, the presence of OA results in a sustained activation of gene expression, as well as in a hyperphosphorylation of a suppressor gene products (Foulkes et al. 1983; Ingebritsen & Cohen, 1983; Ingebritsen et al. 1983; Haystead et al. 1989; Cohen et al. 1990; Nishiwaki et al. 1990; Fujiki & Suganuma, 1993).

Microcystin

Microcystin-LR (MC) was isolated from colonial and filamentous algae and cyanobacteria (Fujiki & Suganuma, 1993). It is a member of the OA class of compounds, but its chemical structure is, to a certain degree, different than that of OA and calyculin-A. Unlike OA, MC appears to be equally effective in blocking PP1 and PP2A. As explained above, MC is slightly more potent in blocking PP1 in comparison to OA and calyculin-A (IC_{50} for the inhibition of PP2A by MC is 2 nM). Moreover, the compound also shows a minimal inhibitory potency towards protein phosphatase 2B (PP2B) or calcineurin. There is also evidence that MC is a tumour promoter and that it could induce tumours in rat liver, initiated with diethylnitrosamine (Fujiki & Suganuma, 1993).

Biochemical analyses have shown that MC binds to the same binding site as OA. In the studies with lipid bilayers OA and MC have been tested whether they permeate the cell membrane. It was shown that while OA was freely permeable, MC was membrane impermeable. Supposedly, this can be an advantage in the present work using a whole-cell technique, where MC, included in the recording pipette, will be in the concentration applied. On the other hand, over a period of time, OA could leak out of the cell and partly disintegrate to its by-products, that could, in turn, reduce its action on protein phosphatases (Frace & Hartzell, 1993).

Calyculin-A

Calyculin-A, is a substance isolated from a marine sponge *Discodermia calyx*. The drug binds to the OA binding site on protein phosphatase 1 and 2A with equal potency, IC_{50}= 2.5 nM (Fujiki & Suganuma, 1993). In comparison to the action of OA, calyculin-A is slightly more potent blocker of PP1, but less potent on PP2A.

1-norokadaone

Another member of the OA group of analogues is 1-norokadaone (1-NO). 1-NO is a synthetic compound that binds to the OA binding site and has similar chemical structure to OA. However, the major difference between these two compounds is that 1-NO has no effect on protein phosphatases. Therefore, it is used in investigations as an inactive analogue of OA.

Protein phosphatase 2B (calcineurin) inhibitors

Protein phosphatase 2B (PP2B) or calcineurin, is completely dependent on Ca^{2+} for its activity, in the presence or absence of calmodulin (Cohen, 1989). It has been proposed that Ca^{2+} ions enter the cytosol via Ca channels, bind to calmodulin and this complex activates PP2B. It appears that this phosphatase then activates Ca^{2+}/calmodulin-dependent protein kinase II (Ca^{2+}-CaM-PKII) (Liu et al. 1991; Brewis et al. 1993). PP2B is practically insensitive to OA and MC and for its action does not require Mg^{2+}, unlike protein phosphatase 2C (Brewis et al. 1993).

A high concentration of PP2B in the brain suggests that this phosphatase may have a number of substrates there. However, the exact function of calcineurin and its pathway in regulation of intracellular processes is still not understood.

It has been reported that some putative immunosuppressant drugs act as potent and specific blockers of PP2B (Schreiber, 1991; Neuhaus et al. 1994). Tacrolimus or FK 506 has been shown to be specific inhibitor of PP2B (Liu et al. 1991; Jayaraman et al. 1992; Schreiber & Crabtree, 1992). Moreover, it has been shown that the action of FK 506 to block calcineurin, depends on the presence of the immunophilin FK-binding protein (FKBP) (Liu et al. 1991).

Effect of protein phosphatase inhibitors

It is known that many different processes in cells depend on phosphorylation. There is a strong indication that protein phosphorylation regulates the efficacy of synaptic transmission, both by controlling the release of neurotransmitters from presynaptic nerve terminals and by modulating the sensitivity of receptors in the postsynaptic membrane (Smith & Augustine, 1988; Huganir & Greengard, 1990; Sihra, 1993; Anholt, 1994; Chavez-Noriega & Stevens, 1994). Presumably, at rest,

a state of phosphorylation/dephosphorylation balance is maintained (Armstrong & Eckert, 1987; Hescheler et al. 1988; Artalejo et al. 1992a; Frace & Hartzell, 1993). It appears that this balance depends on many factors and much of its complexity is not yet understood. It is possible that this equilibrium is kept at two levels. Firstly, some researchers have pointed out to the existence of basally active phosphatase(s) and, subsequently, protein kinase(s). Indeed, some evidence has been given in support of such idea, see later. Secondly, reports have indicated that an inhibition of specific phosphatases potentiates already activated Ca currents (Hescheler et al. 1988; Artalejo et al. 1992a; Frace & Hartzell, 1993; Werz et al. 1993; Wang et al. 1994).

Thus, it was important to examine if there really is any basal activity of protein kinases in cells, and, secondly, whether ion channels have to be in a phosphorylated state in order to open. As it will be shown, the evidence is very often confusing and controversial, depending on the cell population and the protocols employed in the study. Mironov and Lux (1991) used acutely isolated rat hippocampal pyramidal neurones and applied the whole-cell patch-clamp technique. In control conditions, several minutes of perfusion with the intracellular solution were needed for a full development of HVA Ca current. The group argued that, at rest, the HVA current was masked, due to the activity of basally active phosphatases. When they blocked PP1 and 2A with OA, the development of Ca current occurred faster. A similar phenomenon was observed in the presence of the calcineurin inhibitor trifluoperazine. That would imply that PP1, PP2A and/or PP2B at rest suppress the HVA currents in these neurones.

Further evidence that a basally active phosphatase maintains the equilibrium in cells was shown by Frace and Hartzell (1993). In cardiac myocytes the basal Ca current reflects basal phosphorylation activity, suggesting that the channel can pass current when in the dephosphorylated state. They went on to show that PKA was not responsible for the phosphorylation of Ca channels under basal conditions. Internal

perfusion with PKI, a protein kinase A inhibitor, had no effect on basal Ca current. It is, however, possible that other basally active protein kinase(s) mediate this action. Unfortunately, they were unable to identify the protein kinase. They showed that PKA, PKC, tyrosine kinases and calcium-calmodulin kinase II were not involved in the process. It appears that identification of the kinases will be a very complicated task. Hunter (1987) argued that mammalian cells are capable of expressing over fifty different serine/threonine kinases. Therefore, it is indeed possible that other, yet unknown, kinases are involved in the modulation of the ion channels.

Potentiation of Ca current by OA or MC in cardiomyocytes was interpreted as an effect of phosphorylation as it depended on intracellular ATP. In the absence of ATP no effect of OA or MC on the Ca current was observed. Frace and Hartzell showed that calcineurin, a calcium dependent phosphatase, was not involved, because the internal Ca was well buffered.

Furthermore, PKA had no effect on the enhancement of Ca current amplitude induced by OA or MC. Forskolin and cAMP produced an additional increase of the current amplitude in the presence of saturating doses of MC and OA (Frace & Hartzell, 1993).

Several hypotheses emerged from these observations: (1) Is it possible that OA increases Ca current via a different mechanism and does not involve inhibition of dephosphorylation. This is probably not true, because, other groups (Hescheler et al. 1988; Chen et al. 1990; Elmslie et al. 1993) have also shown that the effect of OA and MC is lost in the absence of ATP. However, so far there is no firm evidence to show that these two compounds have a direct effect on protein kinase activities. (2) The potency of the drugs might be influenced by their accessibility to the phosphatases. It was shown that MC is slightly more potent than OA. This observation might be due to their differences in binding affinity (Bialojan & Takai, 1988). (3) It is possible that other, yet unidentified phosphatase regulates Ca channel activity.

Unlike Frace and Hartzell, other scientists (Reuter, 1983; Chad & Eckert, 1986; Armstrong & Eckert, 1987; Chen et al. 1990) have reported that phosphorylation is a "conditio sine qua non" for Ca channels opening. In intact cardiac myocytes (Reuter, 1983) and dialysed molluscan neurones (Chad & Eckert, 1986) it was observed that dephosphorylation of Ca channels leaves the channels in a state from which they do not open in response to membrane depolarisation. The first group to give stronger evidence that Ca channels require to be phosphorylated to open, was that of Armstrong and Eckert (1987). They used cell-free membrane patches in GH_3 cell line from a mammalian pituitary tumour. Using a single-channel recording technique, they exposed the cytoplasmic surface to purified enzymes in the absence of a complex intracellular regulatory mechanism and activated two classes of Ca channels. It was clearly shown that at least one group of the channels, the dihydropyridine sensitive class, had to be phosphorylated to open, following the membrane depolarisation. Furthermore, they observed that the decrease of the channel activity over time was smaller in the presence of 1 mM ATP. Biochemical studies have previously shown that the putative protein of the DHP sensitive Ca channel, purified from skeletal muscle, contains a protease- and phosphatase-sensitive cAMP-dependent phosphorylation site on one of its subunits. They suggested that because cAMP-dependent kinase in brain tissue is often membrane bound, it was possible that ATP prevented the loss of the channel activity by serving as a substrate for an endogenous cAMP-dependent kinase isolated with the patch.

Furthermore, Armstrong and Eckert (1987) proposed that dephosphorylation of that site, and not voltage, leaves the channel in inactivated state in the absence of rephosphorylation. GH_3 cells are rich in calcineurin, which is possibly membrane bound and associated with the cAMP-dependent kinase, and its activity may be important for channel regulation. In molluscan neurones calcineurin dramatically accelerated inactivation of the Ca current (Chad & Eckert, 1986).

Interesting observation was reported by Artalejo et al. (1992a). In bovine

chromaffin cells, this group characterised a novel Ca current, which is normally silent but can be activated by large pre-depolarisations or by repetitive depolarisations to physiological potentials. The current could be additionally stimulated by an activation of PKA; H-7, a non-specific PKA and PKC antagonist, and PKI 14-24 amide, a more specific PKA blocker, partially reduced Ca current. It appears that these compounds did not block the current directly, because in its presence, BayK 8644, a DHP agonist, still potentiated the current. On the other hand, the Ca current was suppressed in the presence of inhibitors of protein phosphorylation or by an injection of phosphatase 2A into the cells. However, when the cells were dialysed with OA, the current was dramatically potentiated, but was not additionally increased in the presence of prepulses. The phosphorylation of the channels was voltage-dependent and could be one of the mechanisms by which membrane potential modulates ion channel activity. These observations suggest that prepulses or repetitive depolarisations can recruit Ca channels by promoting their phosphorylation. Recovery from facilitation involved a dephosphorylation step. It appears that although PKA can potentiate Ca current, it is not directly involved in the action of prepulses. There is a possibility, that an "unidentified kinase" and PKA act on the same site on the Ca channel that is phosphorylated during the prepulse application. The group proposed a model suggesting that depolarisation may change the conformation of the Ca channel, making it a better substrate for protein kinase.

As described earlier, a number of groups (Kodama et al. 1986; Hescheler et al. 1988; Elmslie et al. 1993; Frace & Hartzell, 1993; Ono & Fozzard, 1993; Werz et al. 1993; Wang et al. 1994) have shown that OA applied to many different cell types modulated Ca currents. Actions of the drug on the currents are various. In heart muscle, OA increased the duration of the action potentials and enhanced the contractions of cardiac papillary muscle (Kodama et al. 1986). The positive inotropic effect of OA was not abolished following a treatment with TTX or ryanodine. Moreover, OA increased the amplitude of the end-plate potential at frog

neuromuscular junction (Abdul-Ghani et al. 1991), and modulated synaptic transmission (Swain et al. 1991). Elmslie et al. (1993) suggested that phosphorylation of the channels was necessary to maintain the G-protein mediated inhibition of Ca current following an application of norepinephrine. Hescheler et al. (1988) and Werz et al. (1993) reported that OA potentiated Ca current, increased inactivation kinetics and slowed "run-down" of the current in guinea-pig cardiomyocytes and frog sympathetic ganglion neurones, respectively.

Abdul-Ghani et al. (1991) suggested that phosphorylation could regulate the release of neurotransmitters. Addition of 1 μM OA into the extracellular solution doubled the amplitude of the end-plate potential at frog neuromuscular junction. The effect was presynaptic and the transmitter release reversibly increased following the nerve stimulation. At the lobster neuromuscular junction, OA increased both excitatory and inhibitory synaptic responses following nerve stimulation. OA had no effect on the membrane potential in either preparation. Similar observations were reported by Swain et al. (1991) at crayfish neuromuscular junction where the effect of OA was dependent on its concentration. At lower doses (0.5-5 μM) the effect was exclusively presynaptic and an increase in transmitter release was observed. However, with higher doses of OA the effect was postsynaptic. This might suggest that transmitter release is constantly regulated by phosphorylation of the membrane proteins on the nerve terminals.

It appears that phosphorylation of channel proteins is necessary to preserve a neurotransmitter-induced modulation of Ca current by norepinephrine. A simple analysis suggests that if an activated G-protein acted directly on the Ca channel, coupling of the receptor with the channel should require only GTP. However, there is an evidence to suggest a requirement for the presence of intracellular ATP. During long intracellular recording the preservation of Ca current modulation by NE was sustained only in the presence of both ATP and GTP in the recording pipette in frog sympathetic neurones (Elmslie et al. 1993). A need for both ATP and GTP suggested

the existence of an intracellular phosphorylation step that occurred simultaneously or around the time of receptor-channel coupling.

In many different cell types OA had no effect on the inhibition of Ca current induced by neurotransmitters. For example, OA did not prevent the inhibition of Ca current by NE (Elmslie et al. 1993; Werz et al. 1993) in sympathetic neurones. In the same neurones, inactivation was stronger in the presence of OA (Werz et al. 1993). However, Yakel (1992) showed a reduction in the inactivation rate in the presence of OA in snail *Helix aspersa* neurones. The effect was not observed with 1-norokadaone, an inactive OA analogue.

Calcineurin may also play a role in the modulation of Ca currents. A train of depolarising pulses increased Ca current in smooth muscle cells. The strong and reversible potentiation of the current was independent of cAMP stimulation, unlike in adrenal chromaffin (Artalejo et al. 1990) or cardiac (Reuter, 1983) cells, and H-7 had no effect. However, 10 mM BAPTA included in the pipette solution or the inclusion of the calmodulin inhibitory peptide, RS20, blocked the augmentation of Ca current. This observation showed that the potentiation of Ca current can possibly be mediated by the activation of calmodulin-dependent protein kinase II (McCarron et al. 1992).

Armstrong and Eckert (1987) suggested that the dephosphorylation of Ca channels by calcineurin is responsible for the inactivation of L-type channels. Frace and Hartzell (1993) perfused frog cardiomyocytes with the calcineurin inhibitory peptide, CNIP, and did not observe any particular effect. Very high, nonspecific, concentrations of the drug slowed an increase in Ca current amplitude, accompanied with an increase in inactivation. Yakel (1992) used the same PP2B inhibitor and recorded a significantly reduced rate of inactivation of Ca current in neurones from the snail *Helix aspersa.*

In summary, it appears that strong evidence already exists to suggest that protein phosphatases 1, 2A and 2B are involved in a modulation of Ca current. However, it is unclear whether these phosphatases have a role in maintaining basal phosphorylation/dephosphorylation equilibrium. Furthermore, not all Ca channels have to be phosphorylated in order to open. It appears that in many cell types the inhibition of dephosphorylation up-regulates Ca current. On the other hand, it is not clear whether the regulatory pathway for phosphatases is identical to that used by neurotransmitters to modulate Ca current.

Purpose and aims of Thesis

The main aim of this study was to examine Ca channel currents in dorsal raphé neurones, together with their modulation following activation of the 5-HT_{1A} receptor. Previously, Ca currents in DR neurones have been characterised to some extent (Penington et al. 1991; McAllister-Williams, 1992) and the procedure has been validated here. The work presented confirms the previous observations and examines additional properties and modulation of Ca currents by the 5-HT_{1A} agonist 8-OH DPAT and G-proteins. This study gives the first detailed report of the role of phosphorylation of Ca channels in DR neurones, and of the voltage and temperature sensitivity of Ca current kinetics, in the presence and in the absence of 5-HT_{1A} receptor activation. In addition, the possible involvement of second messengers in modulation of Ca current is touched and briefly discussed.

Following *Chapter 2*, which describes procedures and techniques performed, there are four Chapters which describe the results obtained in this study and their analysis. The first, *Chapter 3*, describes the basic properties of DR neurones, membrane and

action potentials, together with the characterisation of peak Ca currents and their block by cadmium. It has been shown that a whole range of neurotransmitters modulate Ca current in a number of neuronal preparations (Tsien et al. 1988; Kasai & Aosaki, 1989; Cox & Dunlap, 1992; Boland & Bean, 1993). This action of neurotransmitters results in changes in Ca current amplitude, usually accompanied with a change in the current kinetics. Modulation of the peak Ca current and its kinetics following 5-HT_{1A} receptor activation in DR neurones is described in this Chapter.

There is a strong evidence to suggest that G-proteins mediate the action of neurotransmitters on calcium currents in many cell types. For example, it was shown that the $GABA_B$ agonist baclofen inhibited Ca current via G-protein in cultured dorsal root ganglion neurones (Campbell et al. 1993). In DR neurones the effect of G-protein on the peak Ca current was also tested, and its temperature-dependency. The peak current kinetics in control cells, and in the cells perfused with either 8-OH DPAT or GTP-γ-S are analysed in detail, and fitted using mathematical functions, see *Chapter 4*.

Multiple phosphorylation sites on calcium channels have been shown to exist, and it is possible that the effects of transmitters are mediated by a change in the phosphorylation state. Specific inhibitors of dephosphorylation were used, and their effect on the peak Ca current studied, as shown in *Chapter 5*. During the whole-cell voltage-clamp recordings performed in this study, a slow and inevitable "run-down" of Ca currents occurred. This means that calcium current amplitude gradually and irreversibly decreased during recordings. The effect of phosphatase inhibitors on "run-down" was examined, and analysis of Ca current kinetics in the presence and in the absence of 5-HT_{1A} receptor activation was performed, see *Chapter 6*. In addition, the phosphatase inhibitors were tested in the presence of 5-HT_{1A} receptor activation to investigate whether they modulate the same pathway as 8-OH DPAT. Furthermore, more data is obtained to show whether second messengers are involved in the 8-OH

DPAT-induced modulation of the currents in DR neurones or if this is a membrane-delimited pathway.

In the last part of this study, in *Chapter 7*, the results are discussed, and further ideas are proposed about the nature of modulatory pathways of calcium currents. Finally, conclusions about the effects of 5-HT_{1A} receptor activation and role of phosphorylation on calcium currents in DR neurones are drawn.

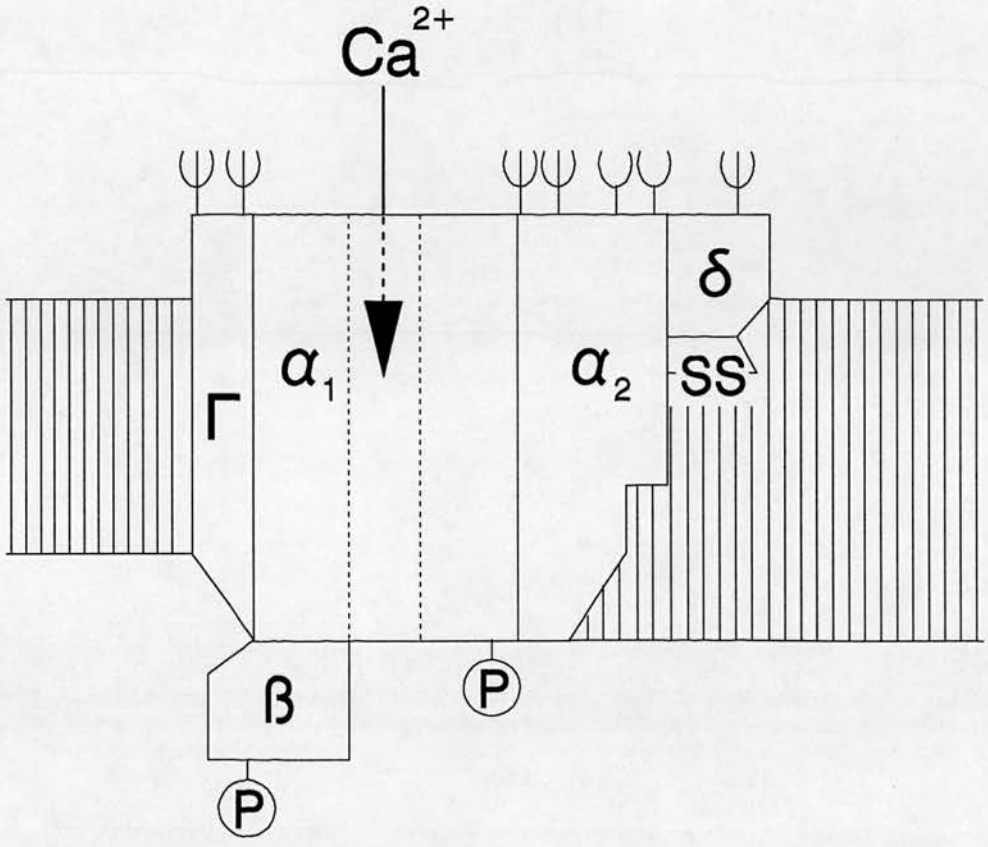

Figure 1.1: Proposed model for calcium channel structure. Calcium channel with subunit structure is schematically shown in an open configuration. Sites for cAMP-dependent phosphorylation (P), disulfide bond, glycosylation and interaction with the membrane are illustrated. Picture is modified from Catterall et al., *Ann.N.Y.Acad.Sci.* 560, 1-14 (1989), and described in the text.

Table 1.1: Modulation of calcium currents by putative neurotransmitters and other substances.

Substance	**Inhibition***	**Neurone type**	**Reference**
Acetylcholine	++	NG108-15	Caulfield et al. 1992
	++	rat hippocampus	Toselli et al. 1989
			Jones et al. 1992
	++	rat SCG	Wanke et al. 1987
Adenosine	+++	chick DRG	Kasai & Aosaki, 1989
	+++	rat DRG	Dolphin et al. 1986
ATP	+++	bullfrog SG	Elmslie, 1992
	++	chromaffin cell	Gandia et al.1993
DADLE	++	NxG	Hescheler et al. 1987
	+	NG108-15	Hescheler et al. 1987
			McFadzean & Docherty, 1989
Dopamine	+++	chick DRG	Marchetti et al. 1986
DPDPE	++	guinea-pig SMN	Surprenant et al. 1990
Dynorphin A	+++	bullfrog DRG	Bean, 1989
	+++	rat SC	Sah, 1990
	+	mouse DRG	Gross & McDonald, 1987
$GABA_B$	++	chick DRG	Deisz & Lux, 1985
			Grassi & Lux, 1989
			Cox & Dunlap, 1992
	+	rat DRG	Dolphin & Scott, 1987
Glutamate	++	rat hypothalamus	Zeilhofer et al. 1993

GTP-γ-S	+++	AtT-20	Lewis et al. 1986
	+++	bullfrog SG	Elmslie, 1992
	+++	chick DRG	Grassi & Lux, 1989
			Kasai & Aosaki, 1989
			Marchetti & Robello, 1989
	+++	chromaffin cell	Gandia et al. 1993
	+++	rat DRG	Dolphin & Scott, 1987
			Dolphin & Scott, 1989
	+++	rat DRN	Penington et al. 1991
	+++	rat SCG	Ikeda & Schofield, 1989
Leu-enkephalin	+++	NG108-15	Tsunoo et al. 1986
LHRH	+++	frog SG	Bley & Tsien, 1990
	++	frog SG	Boland & Bean, 1993
			Elmslie et al. 1990
Met-enkephalin	++	guinea-pig SMN	Surprenant et al. 1990
Muscarine	+++	bullfrog SG	Elmslie, 1992
Noradrenaline	++	bullfrog DRG	Bean, 1989
	++	chick DRG	Forscher & Oxford, 1985
			Holz et al. 1986
			Marchetti et al. 1986
			Cox & Dunlap, 1992
	++	chick SG	Golard & Siegelbaum, 1993
	++	frog SG	Lipscombe et al. 1989
			Bley & Tsien, 1990
	++	guinea-pig SMN	Surprenant et al. 1990
	++	NG108-15	McFadzean & Docherty, 1989
			Caulfield et al. 1992
	++	rat SG	Galvan & Adams, 1982

	++	rabbit PSG	Akasu et al. 1990
Serotonin	+++	rat DRN	Penington & Kelly, 1990
			Penington et al. 1991
	++	rat SC	Sah, 1990
	+	rat hypothalamus	Koike et al. 1994
Somatostatin	+++	AtT-20	Luini et al. 1986
	+++	NG108-15	Tsunoo et al. 1986
	+++	rat SC	Sah, 1990
	+++	rat SCG	Ikeda & Schofield, 1989
	++	chick CG	Meriney et al. 1994
	++	chick SG	Golard & Siegelbaum, 1993
	++	guinea-pig SMN	Surprenant et al. 1990
Substance P	++	bullfrog SG	Elmslie, 1992

*- degree of inhibition of Ca current: (+++) strong; (++) medium; (+) slight.

Abbreviations: DADLE: D-Ala, D-Leu-enkephalin; DPDPE: D-Pen-enkephalin; DRG: dorsal root ganglion; DRN: dorsal raphé nucleus; LHRH: luteinizing hormone releasing hormone; SC: spinal cord; SG: sympathetic ganglion; SCG: superior cervical ganglion; SMN: submucosal neurone; PSG: parasympathetic ganglion; CG: ciliary ganglion.

Chapter 2 Methods

Preparation of dorsal raphé neurones

Dorsal raphé nucleus, located in a brain stem, contains a large number of serotoninergic neurones with 5-HT_{1A} receptors (Lawrence et al. 1989). Dissociation of the DR cells in the present experiments was based on the method first described by Kay & Wong (1986), with some variations. DR neurones were acutely isolated, which sometimes has an advantage over cultured cells, where various conditions during the cell growth can affect the expression of calcium channels (Boland & Dingledine, 1990; Allen et al. 1993), in addition to possible alterations in the release of neurotransmitters (Kasai, 1992).

Adult male Cob Wistar rats (200-250 g) were decapitated by a guillotine and the cranium removed. Cranial nerves were exposed and dissected and the whole brain was rapidly removed and placed into a continuously oxygenated ice-cold artificial cerebrospinal fluid (aCSF). The tissue was then transferred onto a filter paper and moistened with the cold aCSF solution. The cerebral hemispheres together with the cerebellum were removed with a razor blade. On the remaining part of the tissue a rostral cut was made just caudally of the superior colliculus and that side of the brain was put onto a plastic platform and fixed with a cyanoacrylate glue. To support the ventral surface of the brain, a 4% agar block was glued just behind it and the caudal surface was exposed upwards. This part of brain was then transferred into the plexiglass chamber of a vibroslice (Campden Instruments) and submerged in aCSF bubbled with 95% O_2/5% CO_2 mixture and chilled to 4°C. The tissue was carefully sliced using the vibratome in a caudo-ventral direction at the mesencephalic level of the brain stem. The size of the aqueduct of Sylvius, the superior colliculus and the decussation of the superior cerebellar peduncles were additional markers for the localization of DR nucleus. Three to four 400 μm coronal slices were obtained and rapidly placed on an agar base that was moisted with aCSF. The area of grey matter 2 x 2 mm, just below the cerebral aqueduct and dorsal to the decussation, containing

the clearly visible DR nucleus, was now dissected from each slice using a razor blade. This whole procedure of isolation of the pieces of brain tissue containing DR nucleus took on average 7-10 min. Sometimes, to provide more tissue for the experiments, two rats were used in rapid succession.

The pieces of grey matter containing DR nucleus were isolated and rapidly transferred into 10 ml of a piperazine-N,N'-bis-[2-ethanesulfonic acid] (PIPES) buffered solution and enzymatically treated with 5000 units/ml (0.07%) of trypsin (Type XI). The solution was bubbled with a pure oxygen and the tissue stirred using a magnetic stirrer at a rate just sufficient to keep the pieces of tissue suspended. The incubation lasted for 90 min at 33° C exactly. The pieces of tissue were then washed in the same solution, to remove the trypsin, and kept at room temperature (18-22° C) for at least 60 min while bubbled with 100% O_2. Just prior to the use of the DR neurones (1-8 hours later), two to three pieces of tissue were withdrawn and triturated with three fire-polished Pasteur pipettes in turn with end tips of decreasing size of approximately 1 to 0.5 mm in diameter in Dulbecco's modified Eagle's medium (DMEM). Neurones, now bathed in DMEM, were transferred to a 35 mm Petri dish (Corning) and allowed to settle on the bottom of the dish for 15-20 min. The dish was placed in a specially designed holder on the stage of an inverted phase contrast Nikon Diaphot microscope, see Figure 2.1.

Electrophysiology of dorsal raphé neurones

DR neurones that settled on the bottom of the dish and were "phase-bright" with short dendrites and soma diameter of at least 20 μm were chosen for the experiments, see Figure 2.2.

Borosilicate glass electrodes (Clark Electromedical Instruments) with a filament and standard wall and inside and outside diameter of 0.86 mm and 1.5 mm, respectively, were made using a List-Medical L/M-3P-A puller. For a number of experiments the tips of the electrodes were fire polished. They were mounted on the headstage and lowered to the Petri dish. With the tip of the electrode submerged into the extracellular solution, an Axoclamp-2A amplifier (Axon Instruments) was turned on in the "bridge mode" and the electrode resistance measured. The resistance of the electrode filled with the internal solution, was, on average, 6-11 MΩ (mean= 8.7±0.1 MΩ), n= 129. The tip potential was balanced before bringing the electrode tip in the contact with a cell. Junction potentials, measured in the presence and in the absence of the drugs differed by less than 2 mV.

The tip of the electrode was moved towards the chosen neurone using a Burleigh patch-clamp micromanipulator PCS-250. With a continuous application of current pulses of 0.03 nA and 10 ms in duration at a frequency of 10 Hz, a small voltage deflection was seen when the tip touched the cell. Negative pressure was immediately applied by mouth or with 1 ml syringe to the suction electrode until a seal between the electrode glass and the soma membrane was formed. The seal resistance was on average of 5.8±0.4 GΩ (n= 113). At that point a few cells went into a whole-cell formation spontaneously, n= 16. In other cells a further pulse of negative pressure was required to disrupt the cell membrane and convert the mode from the cell-attached to the whole-cell configuration. It was observed that the "gigaohm-seals" were obtained more easily in an external solution containing calcium and for that reason a calcium Tyrode solution was used. When the whole-cell configuration was obtained the calcium Tyrode solution was replaced with a standard external (recording) solution. Immediately after obtaining a stable whole-cell voltage-clamp mode, hyperpolarising current was applied to hold the membrane potential at -100 mV. At that holding potential, the cell was held for 5 min before any further manipulation was done to equilibrate and the content of the patch pipette dialysed the

neurone. Capacitance neutralisation, gain and phase controls were adjusted to produce optimal clamp efficiency. Under these conditions, a clamp gain of between 1 and 2.8 nA/mV was achieved. One agarose bridge (a 1 mm diameter glass tube containing 4% agar in 3 M KCl) connected the ground well with the main chamber. The reference "ground" electrode was connected to a Ag/AgCl pellet at the bottom of a small well filled with 3 M KCl. In all experiments shown here, test pulses that evoked peak Ca currents were applied from V_H -100 mV to -10 mV, for 150 ms, at a frequency of 0.05 Hz. In the experiments with a prepulse application, the prepulses were intermittently applied 10 ms prior to the test pulse, from the same holding potential of -100 mV to 40 mV, for 100 ms.

Recordings were made with a high input impedance amplifier Axoclamp 2A. Single-electrode voltage-clamping (SEVC) with this amplifier allows both current passing and voltage recording using the same microelectrode. The electrode, after it penetrates the cell, records voltages V_e (the voltage developed on the microelectrode resistance and capacitance by the current I_0) and V_m (change of the membrane potential compared to the resting potential) and samples them in amplifier A1, see Figure 2.3. The sample-and-hold amplifier (SH1) produces the sampled value V_{ms}, that becomes an input to another amplifier A2. When the switch S1 is in the current passing position, the output of A2 flows to a CCS unit (controlled current source), which then injects current into the electrode. This current is dependent and directly proportional to the CCS input voltage, and is not influenced by the electrode resistance.

In the current-passing period, T_i, the current pulses cause V_e to rise and the process is dependent on the capacitance of the wall of the glass microelectrode to the solution and the capacitance at the input of the buffer amplifier. At the end of the current-passing time, S1 switches to the "voltage-recording" position. An input to the CCS unit is zero now, and, therefore, the output is also zero. During this period, the microelectrode records voltage. The amplifier A2 provides negative feedback to

clamp V_{ms} to a value almost equal to the command voltage, V_c. As shown in Figure 2.3 (B), V_{ms} slightly changes around the average value, due to the system, electrode and membrane "noise" sources. Therefore, the difference between V_{ms}(ave) and V_c is a common error of the voltage clamp, that mainly results from the open-loop transfer conductance (G_T). This conductance is a product of the transfer conductance of the CCS unit and the voltage gain of A2.

The circuit could also be switched to the "current clamp" position with the S2 switch. This mode was used to make a "gigaohm-seal" formation and to enter a whole-cell configuration. In that mode, during the current-passing period the CCS unit gets input from a current-command voltage (V_i) and injects the current I_0 into the electrode. V_{ms} is a reliable measure of the changes in the membrane potential during current clamp.

Open-loop transconductance (G_T), the switching period (T), the duty cycle (D), electrode tip resistance, R_e, and the transmural capacitance can all be optimally set up. To some extent, the operation of the system also depends upon the resistance (R_N) and capacitance (C_N) of the membrane that is clamped. There are some practical steps that were taken in the experiments: it was necessary to keep R_e as low as possible, although that might affect a resistance seal, furthermore, a smaller volume of solution in the Petri dish usually helped, and compensating for residual C_e and other capacitance in the system, was done by the capacity compensation (CC) dial. The cycle frequency (1/T) was also adjusted; the range of sample rates was, on average, 8.5-19 kHz with a mean of 14.3±0.26 kHz, n= 129.

The operation of the SEVC could also be described by a series of equations (Finkel & Redman, 1984). As described earlier, each cycle of a period T consists of a period T_i, during which the current is applied to the electrode, and T_v, when no current is applied and voltage is recorded. Thus

$$\mathbf{T= T_i + T_v}$$

and

$$\mathbf{T_i = DT}$$

where D is the duty cycle.

Figure 2.3 shows the clamp operation during the n'th cycle. At the end of the cycle, a sample of membrane potential $V_{ms}(n)$ is stored, as explained previously. The electrode current I(n) flowing during $T_i(n)$ is dependent on the amplifier error voltage $\epsilon_1(n-1)$ at the end of the previous cycle, and by the open-loop transfer conductance G_T. When V_c is the command voltage, then there is:

$$\mathbf{I(n) = G_T\, \epsilon_1(n-1)}$$
$$\mathbf{\epsilon_1(n-1) = V_c - V_{ms}(n-1)}$$

The minimum value of T is limited by the response speed of the microelectrode and of the electronic circuity. The following equation allows a steady-state "equivalent electrode resistance" (R_a) to be defined, where $R_a = V_e(\infty)/I(\infty)$. Thus

$$\mathbf{R_a = \frac{R_e\,(1-e^{-DT1}) * e^{-(1-D)T1}}{1 - e^{-T1}}}$$

where R_a is "equivalent electrode resistance"; R_e is electrode resistance; and D is the duty cycle.

The steady-state error can be reduced by increasing the duration of the current-passing period and by increasing G_T with other stability requirements. If $D \to 0$, and as $R_a \to 0$, the error voltage is:

$$\epsilon(\infty) \rightarrow \frac{V_c}{1 + R_N G_T}$$

Temperature analysis

A temperature controller was used to test the effect of temperature on Ca currents. A small Petri dish on the microscope stage was surrounded by a heat sink with two peltiers, see Figure 2.4. These peltiers regulated the temperature of the heat sink and, consequently, heated and cooled the recording solution with DR neurones. When the outside temperature was more than 5° C warmer than the set temperature, ice-cold water was passed through the copper tubing, which was in a tight contact with the heat sink.

This specially designed temperature manipulator, Peltier Temperature Controller, allowed a smooth transition from heating to cooling and prevented development of any sudden switching events that could cause electrical spikes in the vicinity of the electrode. Also, the control unit was connected to dry cell batteries to eliminate the introduction of 50 Hz mains "noise" into the Faraday cage. The temperature controller was calibrated with a bipolar thermometer (Comark) with the reference thermistor placed in boiling tetramethylsilene and the test thermistor in the centre of the Petri dish under normal operating conditions. The central area of the dish was kept at a fairly constant temperature. In a map produced by Medical Systems Corp., with a set temperature at 20° C, a variation in temperature of no more than 0.5° C in the central area of the dish occurred. The majority of the experiments presented here were done at 20° C exactly, and a number of neurones were additionally held at a range of temperatures from 15° to 30° C. Increases in temperature of 5° C, which was the

minimal step used in the experiments, can be performed in less than 1 min, while decreasing a temperature by 5° C, lasted, on average, 1-4 min. This depended on the room temperature and whether or not ice-cold water was passed across the heat sink. Recordings were normally made 2-3 min after the temperature settled at a new level.

Solutions and drug application

Most solutions used for the dissociation of DR neurones are as in the method described by Kay and Wong (1986) and tested by other workers (Penington et al. 1991; McAllister-Williams, 1992). The composition of the solutions used in the present experiments is shown in Table 2.1. A peristaltic pump was used to deliver an external solution into the Petri dish that contained DR neurones with a rate of 1 ml/min exactly. A "bubble trap" was made in the line to minimise the pulsatile flow and electrically isolate the dish from the pump. The level of solution in the Petri dish was kept constant by a suction device connected to a vacuum pump. This output was placed diagonally opposite the input line that brought the bathing solution into the dish. In addition, 100% O_2 was continuously blown across the dish at a rate of 100-150 ml/min.

As described earlier, calcium Tyrode solution was used to establish the whole-cell mode. Immediately upon obtaining a whole-cell mode, the extracellular perfusion was switched to a standard recording solution that was designed to isolate Ca currents. Sodium was replaced by tetraethylammonium (TEA) and a contribution of sodium currents was additionally eliminated by tetrodotoxin (TTX, see Table 2.2). Furthermore, potassium currents were completely blocked by TEA and also by the addition of 4-aminopyridine (4-AP) to prevent the appearance of the A-current that

these cells exhibit (Burlhis & Aghajanian, 1987; Aghajanian et al. 1990). Barium ions were used as the main charge carriers, instead of calcium. That has certain advantages and disadvantages. The main reason was that in the presence of Ba^{2+}, the "run-down" process, studied in these experiments, was smaller (Dolphin & Scott, 1989). Furthermore, there was no activation of calcium-dependent activities, such as an activation of calmodulin-dependent cAMP phosphodiesterases, activation of ion channels and other intracellular processes in the neurones. Ba^{2+}, additionally, contributed to the inhibition of potassium current (Brown, 1990). On the other hand, the calcium channel kinetics might be affected by the presence of Ba^{2+} (Zhang et al. 1994). It has been reported that inactivation of L-type channels is slowed when Ba^{2+} replaced Ca^{2+} in cardiac cells (Sanguinetti & Kass, 1984; Kass, 1987), smooth muscle cells (Lang et al. 1991), pituitary cells (Williams et al. 1991) and hippocampal neurones (Kay, 1991), but not in mouse cerebellar neurones (Slesinger & Lansman, 1991b; Slesinger & Lansman, 1991a; Haws et al. 1993) or rat DRG cells (Tatebayashi & Ogata, 1992). Akasu et al. (1990) recorded faster tail currents kinetics when using Ba^{2+} ions. For that reason, it is important that the results shown are viewed in that prospective. It should also be clear that where ever "calcium currents" are referred to in the presentation of the results, they were actually "barium currents".

In the initial experiments, that were designed to study basic properties of DR neurones, intracellular solution contained (mM): K^+ gluconate 84, HEPES 10, MgATP 2, KCl 38, EGTA 11, KOH 33, $CaCl_2$ 1, GTP 0.3, and pH was of 7.3. In all other experiments, a standard intracellular solution was made in a fashion to optimize Ca current isolation, to block the remaining potassium and possibly sodium currents and to minimise "run-down". TEA was included in the internal solution to eliminate potassium current and MgATP to help to reduce "run-down" (Elmslie et al. 1993). This group also reported that MgATP, together with GTP, helped to maintain the modulation of Ca current by norepinephrine in sympathetic ganglion neurones. Therefore GTP was also added to the solution. Intracellular calcium was buffered by

the inclusion of ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA). EGTA, as a chelator, might not be as fast and sensitive to pH as another buffer 1,2-bis(2-aminophenoxy)ethane N,N,N′,N′-tetraacetic acid (BAPTA), but that appeared not to affect the experiments (McAllister-Williams, R.H. - personal communication). Potassium was replaced by trizma, a large impermeant ion, that was more suitable to minimise "run-down", than a more conventional caesium based solution. The recording pipettes also contained phosphate as the main anion, rather than chloride. Chloride is known to have several disadvantages, such as an interaction with proteins that stabilize plasma membranes (Inoue et al. 1976). Another advantage was that with phosphate included in the standard internal solution, the recordings were more stable and lasted for up to 90 min.

The standard intracellular solution was usually made at the beginning of a week and used for that week only. Ca Tyrode and standard recording solutions were made fresh every day, or every second day. When they were stored overnight, they were kept refrigerated at 4° C. No deterioration or the presence of particles in the solutions were noticed. On the day of an experiment, the solutions were taken out of the refrigerator and left at a room temperature. The standard intracellular solution was kept in a deep-freezer until 3 hours prior to the recordings, drawn up into a 1 ml syringe and kept on an ice bed. The syringe with the filling was allowed to warm up to room temperature 10 min before the cells were tested and then returned to the ice bed. The recording pipettes were filled via a 0.1 μm filter (Acrodisc, Gelman Sciences) that was put in between the syringe and a fine tube pulled with heat to inject the solution into the pipette.

An optimal cell viability was achieved with a pH of the standard recording solution of 7.3, whereas for the standard intracellular solution pH was adjusted to 7.2. Osmolality was finely adjusted with sucrose. The recording solution was calibrated to 320 mOsm and internal to 310 mOsm. Hypoosmotic pipette solution probably

helped to reduce cell swelling. On some occasions DR neurones did swell; that was observed visually when the cell suddenly became spherical, and sometimes an increase in the current amplitude occurred. When a cell became spherical due to swelling it was lost or the recording was stopped and not included in the analysis.

Drugs were added either to the internal solution or perfused into the external solution, see Table 2.2. Guanosine 5'-O-3-thiotriphosphate (GTP-γ-S) and guanosine 5'-O-2-thiodiphosphate (GDP-β-S) were included in the pipette solution at the time it was made up. Okadaic acid, 1-norokadaone, microcystin-LR, calyculin-A, staurosporine, H-7, forskolin, FK 506 and FK binding protein were dissolved in an anhydrous N,N-dimethyl-formamide (DMF) and deep frozen. All compounds were diluted to their final concentration and added to the pipette solution just prior to the start of recordings. In a few experiments, OA was placed in the back of the recording pipette ("back filling") and the tip of the pipette was filled with a standard internal solution. This method was used to test the time OA needed to dialyse the neurones. DMF (0.5%) has several advantages over more often used dimethyl sulphoxide (0.5%, DMSO) and ethanol (0.2%). Control experiments in DR neurones showed that DMSO and, to a lesser degree, ethanol, significantly potentiated the peak Ca currents in the absence of any other application, see Figure 2.5. On the other hand, DMF did not affect the peak Ca current amplitude, or the effect of forskolin to increase intracellular cAMP, see Figure 2.6. In control experiments, forskolin, dissolved either in DMF or in DMSO, was shown to stimulate cAMP levels in a protein-binding assay (Armstrong et al. 1985).

5-HT, 8-OH DPAT and cadmium were dissolved and applied into the standard external solution. All were made up as stock solutions at 10 mM and kept frozen. Each day, the drug was diluted to its test concentration with the external solution. A short and rapid administration of agonists was possible via a broken glass pipette, with a tip diameter of about 5 μm, positioned 50-100 μm from the cell. The pipette

was placed in a holder and slight positive pressure was produced. It was possible to view the solution flowing out of the pipette to the studied cell. In the experiments where only the external solution was perfused in the same way to the vicinity of the neurones, no change in Ca current was recorded. When the compounds, e.g. cadmium, had to be applied for a longer time, the drug was included into the external solution and perfused into the Petri dish with DR neurones. The externally applied drugs were removed from the bath by a withdrawal of the pipette and simultaneous bath perfusion with drug-free external solution.

A reduction and enhancement of Ca current is expressed as a percentage of the change of the control Ca current amplitude (100%) obtained in the standard recording solution and in the absence of any drugs. All results are expressed as the means ± S.E.M. For evaluation of the statistical significance of the results, Student's *t*-test was used with a level of significance as $p < 0.05$.

Data analysis

Currents were measured in respect to the zero current level and Ca current was evoked as a peak inward current. Ca channel currents ran down slowly with time. As described earlier, after obtaining a whole-cell voltage-clamp mode, a neurone was perfused in Ca Tyrode solution for 5 min and held at holding potential of -100 mV, without eliciting currents. Correspondingly, the time course of the peak Ca current amplitudes shown in the graphs is shown with no currents evoked in the first 5 min after establishing the whole-cell mode. Thereafter, every second peak is shown in the graphs. An inevitable "run-down" that occurred during recordings was measured from the time the peak Ca current amplitude was recorded to 30 min later. It is also

sometimes shown as a percentage of "run-down" per minute, although this is not an optimal reference, because the "run-down" was not linear. However, the data were not analysed if "run-down" was rapid and the same cell lost during the first 30 min of recording.

Current and voltage data had a direct input, after filtering at 1 kHz, onto a video cassette recorder Sony SL-F30 via a Sony PCM-701 digital audio processor. The data were then digitised at 5 kHz and transferred to a DELL PC386 IBM-compatible computer, using CED 1401 Electrophysiology Package V6.0 - Current and Voltage Clamp Analysis (Cambridge Electronic Design, Cambridge). Linear components of capacitance and leak currents were subtracted digitally from all records shown. Leak and capacity subtraction was calculated from small test pulses that elicited no active current, and then scaled linearly. Leak sweeps consisted of ten hyperpolarising (10 mV) test pulses that were later averaged. The leak currents were scaled to the appropriate size and then subtracted from the individual current records. Data was not accepted unless voltage errors from series resistance remaining after partial compensation were less than 5 mV. To eliminate residual capacity artifacts after leak subtraction, current data points 0.4-0.6 ms following a depolarising voltage step have been left blank in the figures. Moreover, because of an incomplete capacitance subtraction and sensitivity of the clamp system, the first few tenths of milliseconds of tail currents were difficult to analyse and there was a possibility of making a considerable error in measuring current kinetics. It was, therefore, decided that the tail current amplitudes and kinetics will not be included in the presentation. For the same reason, fast activation kinetics with time constants of less than 1 ms were not suitable for analysis. All voltage traces shown here are as recorded.

Ca current activation, inactivation and deactivation were fitted using theoretical curves in a Newfit V3.0 programme, partially written in our laboratory (McAllister-Williams, 1992). Fitting of theoretical curves was by means of the

iterative "Amoeba" algorithm (Press et al., 1992) utilising the downhill simplex method in multidimensions of Nelder and Mead (1965). This algorithm involves creating a multidimensional object with one more vertex than unknown constants, whose volume represents the sum of the squares of the difference of the data points from the estimated curve on that iteration. The procedure adjusts the vertices to minimise the volume. Once a minimum is reached, values for the constants are returned. To prevent the detection of false local minima, values obtained were multiplied by 0.1 and 10 and returned to the procedure as starting points for a re-run. This continued until the final sum of the squares of the difference of the re-run varied from the previous one by less than 0.001%. This usually took 2-4 runs of the procedure, though occasionally no definite minimum could be found, and so a maximum of 8 runs was set to prevent the programme being in endless calculations.

Fitting of the curves was averaged to every second point to mostly reduce "noise" and number of iterations. This did not affect the time constants obtained. As an example, a current trace was digitised at 5kHz and forward averaged every 2 points. The calculated activation time constant was of 2.35 ms for a single exponential. If the same data were digitised at the same frequency and all 824 points of the current trace included in the analysis, the time constant for a single exponential was 2.36 ms. The difference was not significant and the error was no more than 0.5%. To double check possible variations all calculated curves were superimposed onto the real data and visually examined to exclude false fitting points.

The current kinetics were fitted with both single and double exponentials. The data were compared and the time constant with the best fit recorded. Single exponential function has the form

$$\mathbf{I_m(t)= A + B*exp^{(-t/\tau)}}$$

whereas a double exponential was calculated using the following equation

$$\mathbf{I_m(t) = A + B*exp^{[-t/\tau(f)]} + C*exp^{[-t/\tau(s)]}}$$

$I_m(t)$ is the membrane current at time *t*; A, B and C are constants; τ, τ_f and τ_s are time constants.

Furthermore, the activation of Ca currents was also fitted using Hodgkin and Huxley m^1 - m^4 models (Hodgkin & Huxley, 1952).

$$\mathbf{I_m(t) = I_{max} * [I_\infty-(I_\infty-I_0)*exp^{(-t/\tau)}]^x}$$

I_{max} is the maximum inward current; I_∞ and I_0 are the final and initial values of I_{max}; x is an integer that describes the fits to this equation as m^1, m^2, m^3 or m^4.

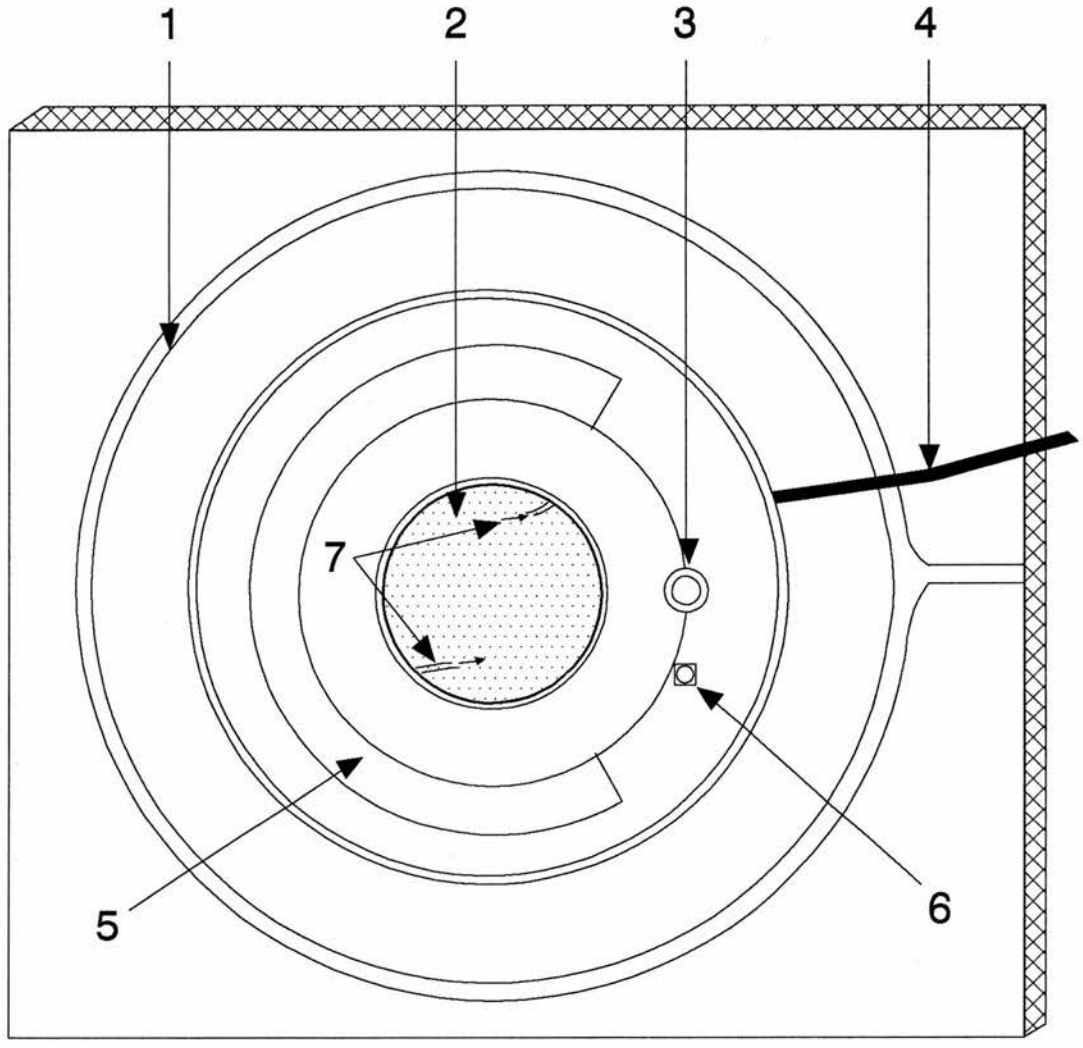

Figure 2.1: Schematic view of a Petri dish placed on the stage of the Nikon Diaphot microscope. Petri dish is positioned on the centre stage of the microscope. Elements for temperature control and tubing for gas and solutions are shown here and described in the text. 1. copper tubing, 2. Petri dish, 3. Ag/AgCl pellet and ground well, 4. temperature controller power cable, 5. metal plate, 6. gas inlet, 7. solution inlet and outlet.

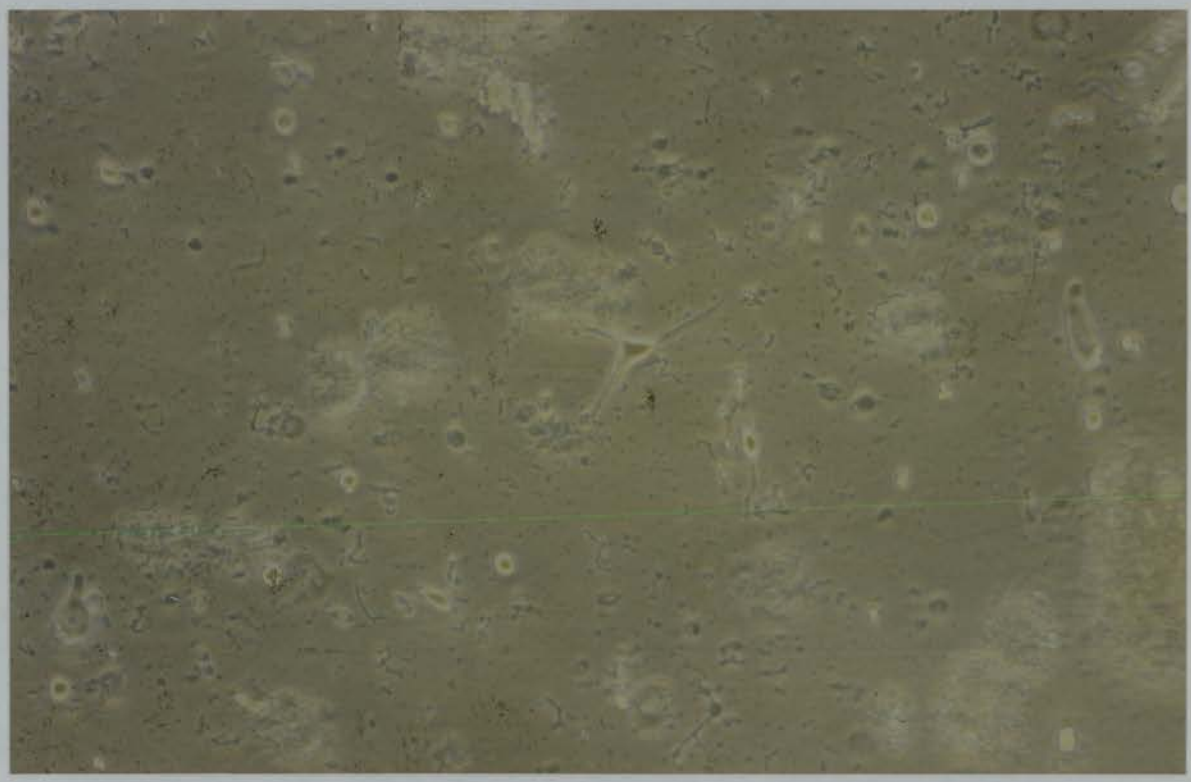

Figure 2.2: A phase contrast photomicrograph of a typical dorsal raphé neurone. Dorsal raphé neurones were isolated using mechanical and enzymatic dissociation. Healthy cells with triangular "phase-bright" somas and the shortest diameter of at least 20 μm were used for the whole-cell recordings. Dendrites were short and truncated to obtain an optimal space clamp. Bar represents 50 μm. (Kindly provided by Dr. J. Lawrence).

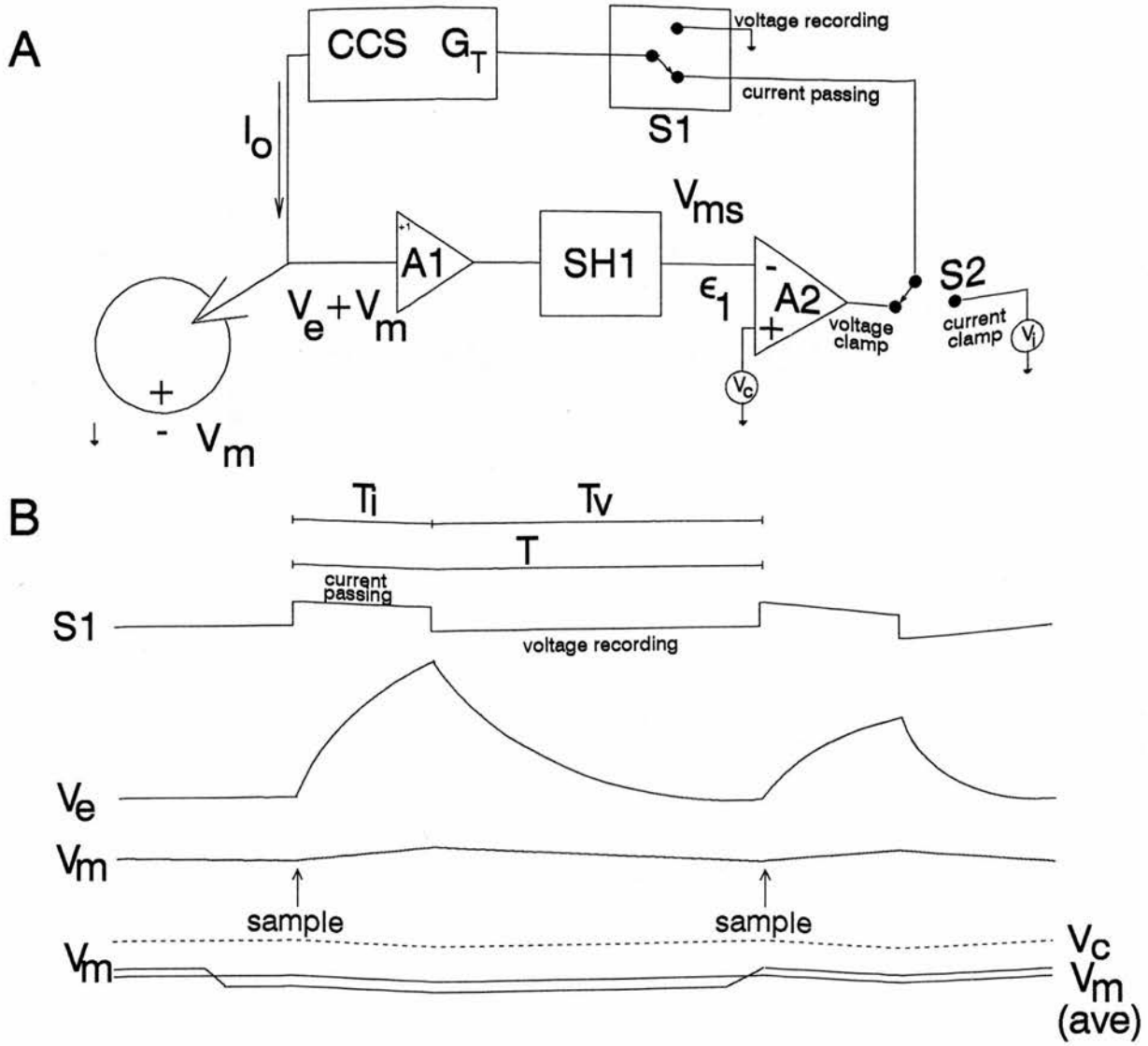

Figure 2.3: Scheme of the operation of a single-electrode voltage-clamp. A block diagram (A) and timing diagram (B) describe the principle of current passing and voltage recorded events. Switch S1 is closed during the current passing period. The microelectrode voltage (V_e) is shown as an exponentially increasing voltage during the current-passing, and an exponentially decreasing during the voltage-recording period, when S1 switch is grounded. The membrane potential (V_m) increases linearly during the voltage recording period. The sum of V_e and V_m is sampled at the times indicated in (B). V_{ms} is compared with the command voltage (V_c), and V_{ms} (ave) is the average value of V_{ms}. Modified from Finkel and Redman, *J.Neurosci.Meth.* 11, 101-127 (1984).

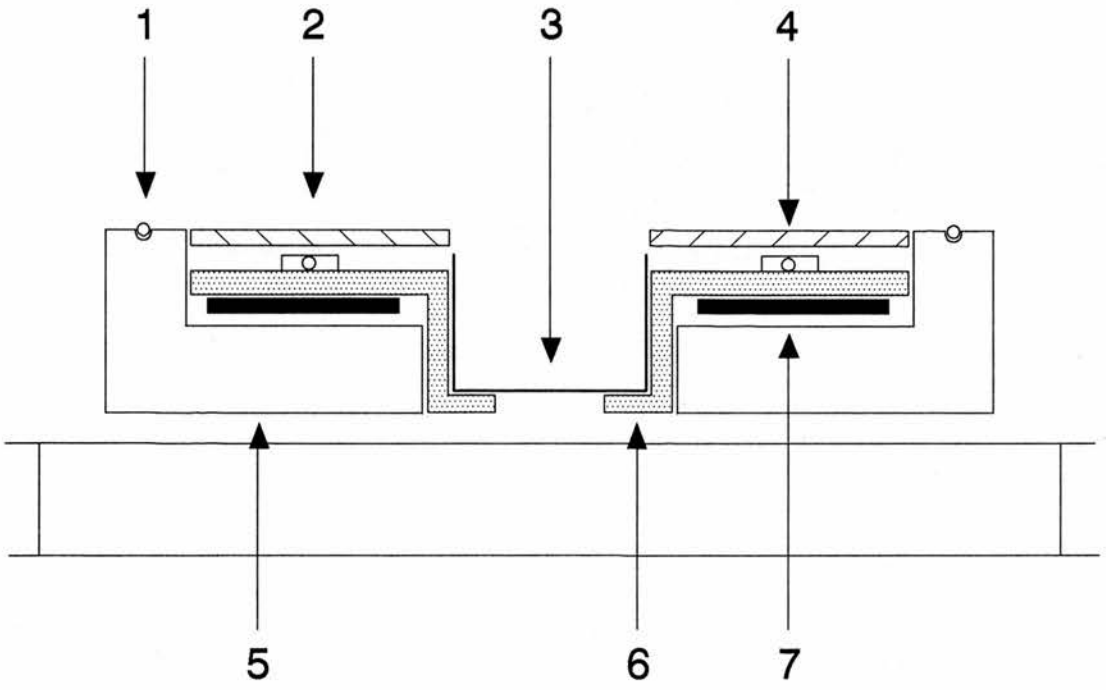

Figure 2.4: Scheme of a cross-section view of Peltier temperature controller. Temperature controller was used to test the effect of temperature on Ca channel current. The scheme of the controller, mounted on the Nikon Diaphot microscope stage, is described in the text. 1. copper tubing, 2. lid, 3. Petri dish, 4. perfusion tube, 5. heat sink (1), 6. heat sink (2), 7. peltier device.

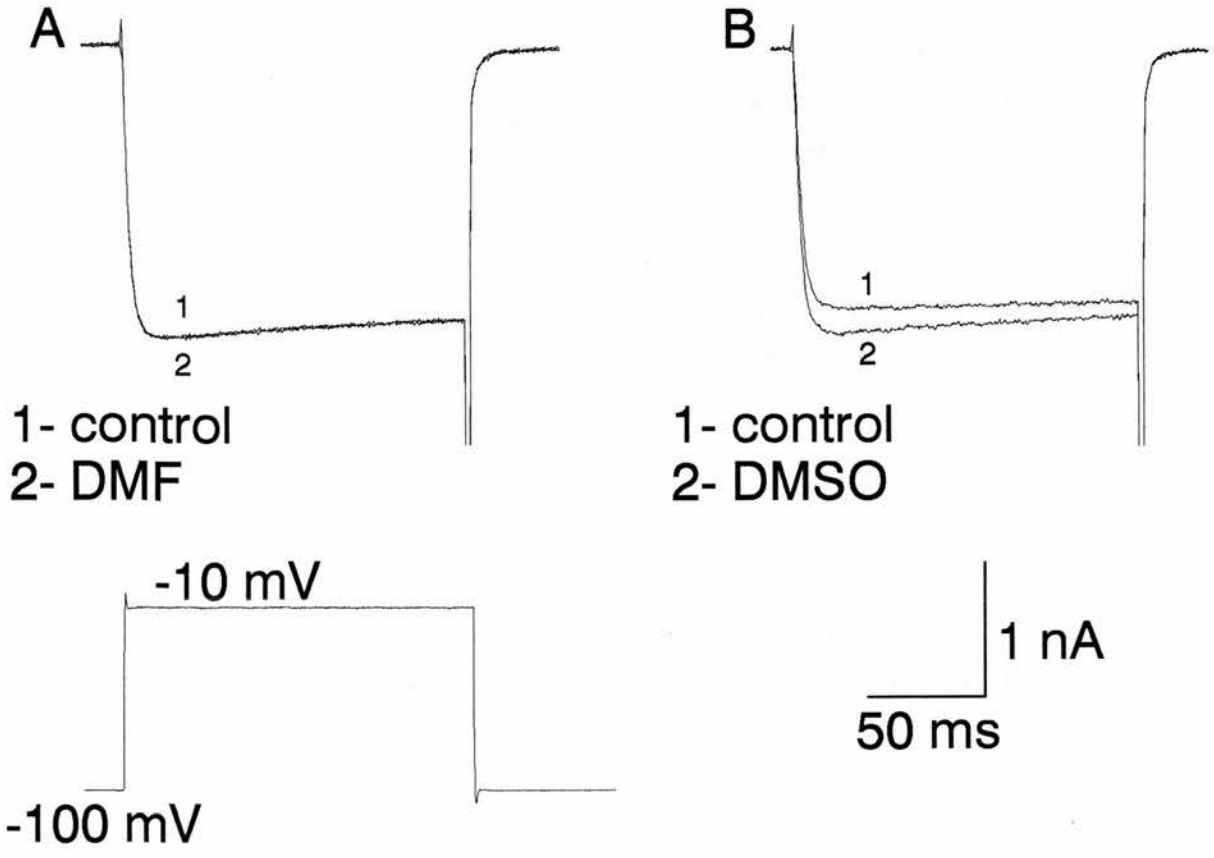

Figure 2.5: Differential effects of DMF and DMSO on the peak calcium current. In a typical DR neurone, DMF was included in the recording pipette and had no effect on the peak current, n= 3 (A). With inclusion of DMSO in the recording pipette, the peak current was enhanced, on average, to 109% of control, n= 3 (B). The peak current was elicited with a voltage step to -10 mV from a holding potential of -100 mV, for 150 ms (lower panel). Currents are leak and capacity subtracted. Standard internal and external solutions were used. Cells 0605931 (A) and 0505933 (B).

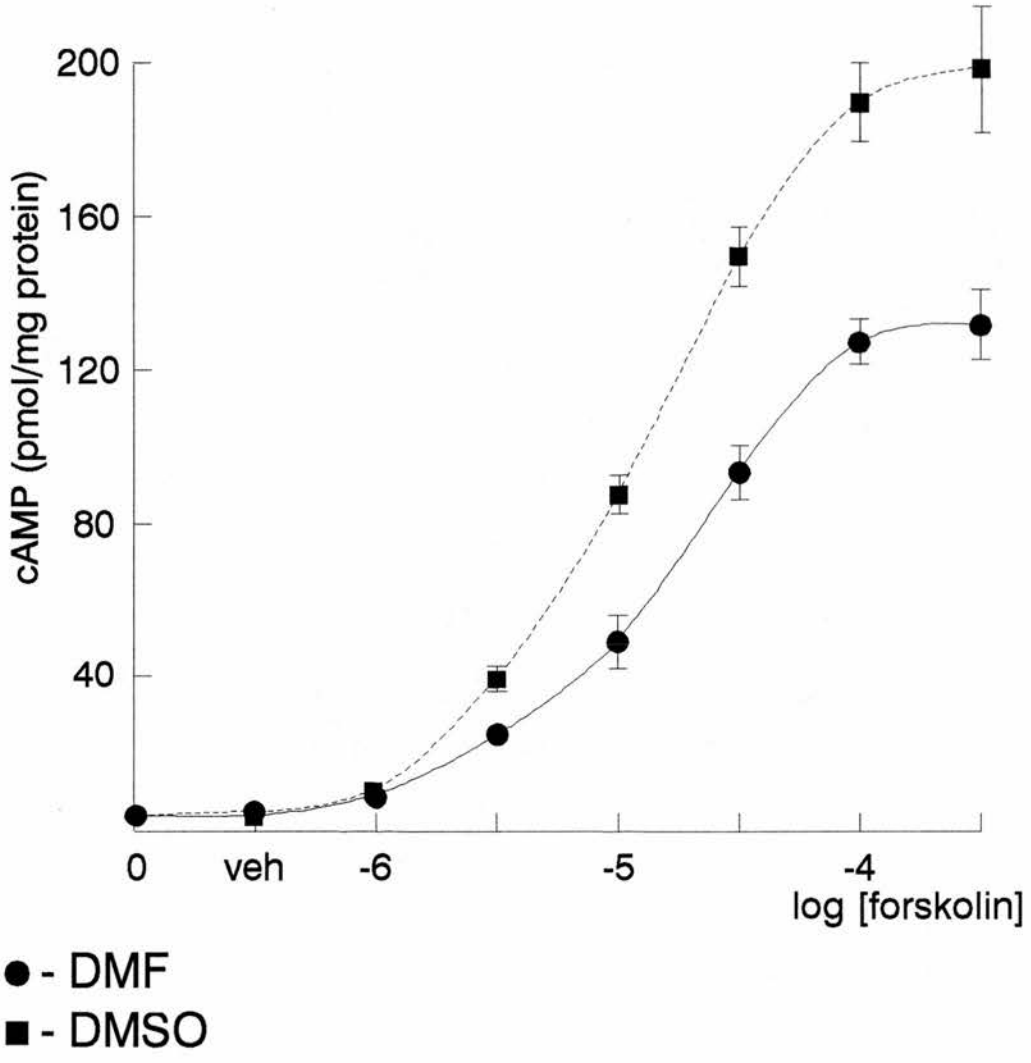

Figure 2.6: Effect of DMF and DMSO as vehicles for forskolin-induced cAMP production in rat cerebral cortical neurones. Forskolin stimulated cAMP production, that was measured using a protein-binding assay, see text. Dose response curves for forskolin dissolved in either DMF (●) or DMSO (■) are shown. The final concentration of both DMF and DMSO was 3.3%, and the neurones were incubated for 5 min at 37°C. Data represent the mean ± SEM of four experiments. (Kindly performed by Mr. H. Ito).

Table 2.1: Table of solutions used in the experiments for dissociation and recordings of dorsal raphé neurones.

	aCSF	**PIPES**	**Ca Tyrode**	**External**	**Internal**
NaCl	126	120	138		
KCl	5	5	1		
$MgSO_4$	2				
$MgCl_2$		1	1		
$CaCl_2$	2	1	5		
$NaHCO_3$	26				
NaH_2PO_4	1.25				
glucose	10	25	10	10	
sucrose			15	20	30
PIPES		20			
HEPES			20	20	
TEA-Cl				138	40
$BaCl_2$				5	
4-AP				5	
Trizma-EGTA					28
Trizma-PO_4					70
MgATP					2
GTP					0.3
pH	7.4 w/95% O_2/ 5% CO_2	7.0 w/NaOH	7.4 w/NaOH	7.4 w/TEA-OH	7.2 w/Trizma base

Table 2.2: Table of drugs used in the experiments and applied to dorsal raphé neurones.

Drug	**Company**	**Concentration**
4-AP	Sigma	
DMF	Aldrich	
EGTA	Sigma	
GDP-β-S	Sigma	
GTP	Sigma	
GTP-γ-S	Sigma	
H-7	Calbiochem	
HEPES	Sigma	
Mg-ATP	Sigma	
PIPES	Sigma	
TEA-Cl	Aldrich	
TEA-OH	Aldrich	
Trizma Base	Sigma	
Trizma PO_4	Sigma	
TTX	Sigma	5×10^{-7} M
5-HT creatinine sulphate	Sigma	10^{-4} M
8-OH DPAT	RBI	10^{-5} M
Calyculin-A	RBI	10^{-6} M
FK 506	Fujisawa	10^{-8} M

FKBP	Fujisawa	10^{-4} M
Forskolin	RBI	10^{-5} M
Microcystin-LR	Sigma	10^{-6} M
1-norokadaone	Calbiochem	10^{-6} M
Okadaic acid	Calbiochem	10^{-6} M
Staurosporine	Calbiochem	10^{-6} M

Chapter 3 Effect of 5-HT_{1A} receptor activation on calcium currents

Basic properties of dorsal raphé neurones

Dorsal raphé neurones were acutely isolated and studied using a whole-cell voltage-clamp technique (Hamill et al. 1981; Kay & Wong, 1987). Basic properties of the neurones, such as membrane and action potential, were examined, with K^+ gluconate containing recording pipettes (see *Methods*). Healthy cells fired spontaneous action potentials under current-clamp conditions, prior to the addition of TTX, see Figure 3.1 (A). This observation confirms the finding (Burlhis & Aghajanian, 1987; Sprouse & Aghajanian, 1988; Pan et al. 1989; Pan et al. 1993) that DR neurones recorded in vitro from raphé midbrain slices, exhibit a slow, regular discharge pattern, suggesting that the cells may have tonic pacemaker properties in the absence of synaptic inputs.

In this experiment, the spontaneous action potentials measured between 70-100 mV in amplitude. Additional action potentials could be elicited by depolarising pulses positive to -60 mV. The frequency of the action potentials varied from cell to cell, and the neurones usually fired at a rate of 5-10 Hz. On an expanded time scale, as in Figure 3.1 (B), note that each action potential consisted of an initial depolarisation, a small shoulder during repolarisation, followed by hyperpolarisation. Thus, it is possible that spikes arose from depolarising ramps, rather than from excitatory postsynaptic potentials (Burlhis & Aghajanian, 1987). TTX (0.5 μM), added to the external solution, abolished the fast component of the action potential and uncovered high- and low-threshold Ca^{2+} action potentials, as shown and discussed before (Penington et al. 1991). The membrane potential of the dissociated DR neurones ranged from -40 to -60 mV.

Penington and Kelly (1990) have shown that at least four types of Ca currents are present in DR neurones. Most of the characterised current is carried through the N-type channels (~40%), and a smaller proportion via L- (~0-20%) and T-type channels. It has been reported so far that many neurones, such as cerebellar granule

neurones, hippocampal CA1 and CA3 neurones, spinal cord and dorsal root ganglia, but not sympathetic neurones, also contain P-type Ca current (Mintz et al. 1992; Mogul et al. 1993; Pearson et al. 1993; Protti & Uchitel, 1993; Bossu et al. 1994). It is important to keep in mind that at V_H -50 mV, where all T-type Ca current is inactivated, an application of ω-CgTx (1 μM) and DHP antagonist nimodipine (1 μM) into the bath, blocked only ~45% of Ca current. Thus, ~55% of the Ca current remains unblocked (Penington et al. 1991).

With 5 mM Ba^{2+} in a recording pipette, as the charge carrier, weak depolarisations from V_H -100 mV to -40 mV for 150 ms, produced a small, ~0.2 nA, rapidly decaying current that almost completely inactivated by the end of the test pulse (data not shown). This was LVA current and had characteristics of T-type Ca current (Fox et al. 1987b; Bean, 1989b; Dolphin, 1991a; Liu & Lasater, 1994b). The size of the current was variable and, in some cells absent. If it was present to a significant degree, a small shoulder was recorded on the I-V relationship. T-type current is also present in other neurones, such as chick (Marchetti et al. 1986; Fox et al. 1987b; Swandulla et al. 1991) and rat (Swandulla et al. 1991; Bossu et al. 1994) sensory neurones, rat cerebellar granule cells (Dolphin, 1991a; Bossu et al. 1994), turtle retinal ganglion cells (Liu & Lasater, 1994a), but in some neurones, such as in rat and frog sympathetic neurones, its presence is variable. Stronger depolarisations elicited larger, high-voltage activated Ca current. In all experiments shown here, only HVA Ca current in DR neurones was studied (n= 129).

Figure 3.2 shows current-voltage relationship in one, representative, DR neurone. Ca currents were elicited from a holding potential of V_H -100 mV to various depolarising potentials for 150 ms, at a frequency of 0.05 Hz. It is assumed that at V_H -100 mV, all available Ca current is de-inactivated. Ca current activation was first observed at test potentials to -40 mV. From this threshold the current gradually increased and from potentials greater than -30 mV the increase in current amplitude was very much steeper. Peak amplitude was always recorded at around -10 mV and

the potential reversed at about 50 mV.

In guinea-pig submucosal neurones and some other neurones, the peak Ca current was recorded at more positive potentials (Surprenant et al. 1990), but not in bullfrog sympathetic ganglion neurones (Boland & Bean, 1993). Furthermore, it was not possible to record the current at test potentials more positive than 80 mV. If the current was elicited with strong depolarising test pulses, to more than 80 mV, large leak currents occurred and the clamp was no longer stable. This might be due to the use of Ba^{2+} as the charge carrier and the use of TEA and not Cs^{+}, in the pipette solution. In other preparations (Boland & Bean, 1993), where Cs^{+} was used in the pipette, a large outward current was recorded, at potentials positive to 40 mV. This group argues that this outward current is carried primarily by Cs^{+} ions flowing through Ca channels, because the current was effectively blocked by ω-CgTx and Cd^{2+}.

"Run-down" of calcium currents

During whole-cell recordings in many different cell types a slow "run-down" of Ca current occurs (Fenwick et al. 1982; Belles et al. 1988; Dolphin et al. 1989; Elmslie et al. 1993; Tiaho et al. 1993). "Run-down" is defined as a slow, but permanent, decrease of Ca current.

"Run-down" is illustrated as a function of time in Figure 3.3. The peak current was elicited with voltage steps from V_H -100 mV to -10 mV for 150 ms, every 20 s. During the first 5 min in a whole-cell mode, the peak current gradually increased and was maximal, on average, in 5-13 (mean= 7.6) min after establishing the whole-cell mode. In that period, the cell was presumably dialysed and outward currents were blocked. Then, the inward current stabilised on average at 1.82±0.08 nA and

gradually, over the time of recording, decreased. It has been shown that the presence of ATP and GTP are crucial to the preservation of the Ca currents during long term recordings (Chen et al. 1990; Elmslie et al. 1993). With Mg-ATP (3 mM) and GTP (0.3 mM) in the recording pipette, the rate of "run-down" in the present experiments was on average 17±4.3%, 30 min after obtaining the maximal peak amplitude, i.e. a decrease of 0.57%/min in the peak current. The recordings were relatively stable and lasted for periods of 30-90 min. Without ATP in a recording pipette, "run-down" was much faster. Furthermore, the recording time was also shorter and lasted for only ~10-22 min, n= 3. During these experiments, the peak Ca current decreased to near zero and the leakage current irreversibly increased, which, presumably, led to a "loss" of the cells.

Ca current was measured at 20° C exactly, and during a voltage step from V_H -100 mV to -10 mV, the peak Ca current was on average 1.82±0.08 nA (n=34). The current consisted of two components, see inset. A transient one with a fast activation constant was followed by a slowly decaying component that represented the inactivation of the current. In majority of DR cells, the amplitude of the current at the end of the test pulse, 1.75±0.05 nA, was similar to that of the transient component. A prepulse was applied 10 ms before the test pulse for 100 ms, from a holding potential of -100 mV to 40 mV. The effect of the prepulse varied. In DR neurones, there is no significant increase in the current amplitude, and sometimes the peak amplitude of the current is slightly smaller than in control experiments, i.e. in the absence of the prepulse. The same phenomenon was reported in guinea-pig submucosal neurones (Surprenant et al. 1990), chick sympathetic neurones (Golard & Siegelbaum, 1993) and bullfrog sympathetic neurones (Elmslie, 1992), but Artalejo et al. (1992a) showed an increase in Ca current amplitude following strong repetitive depolarisations. Mean amplitude of the peak current following the prepulse application in DR neurones was, on average, 1.86±0.07 nA, (i.e. 102±3.6% compared to control).

Kinetics of the peak Ca current were unaltered following a prepulse. Therefore, in DR neurones, it appears that the prepulse had no effect on the peak amplitude nor activation kinetics in control experiments.

Effect of cadmium on calcium currents

Cadmium, Cd^{2+}, recognized as a relatively specific blocker of Ca channels, was also used to characterise the current. Cd^{2+} is known to compete with Ca for binding to Ca channels (Surprenant et al. 1990; Yang et al. 1993). As described earlier, Fox et al. (1987b), Dolphin (1991a) and other groups (Allen et al. 1993; Yang et al. 1993) have shown that HVA channels are more sensitive to Cd^{2+}, than LVA channels. For example, Allen et al. (1993) has reported that in acutely dissociated magnocellular cholinergic basal forebrain neurones of rat, Cd^{2+} at low concentrations ($\leq$ 1 μM) markedly inhibited HVA, but not LVA current. However, at higher doses of Cd^{2+} (3-200 μM), this selectivity was lost and all components of Ca channel current were blocked, with an IC_{50} value of 2 μM.

In the present experiments, internal and external solutions were designed to isolate the current carried through Ca channels, and the peak current was elicited with standard voltage pulses. When the amplitude of the current stabilised, as shown in Figure 3.4, Cd^{2+} (0.1 mM) was applied externally to the cell for 7 min. The drug blocked, on average, 81$\pm$2.8% of the peak Ca current, n= 3. Following wash-out of Cd^{2+}, partial recovery occurred. It was, to some extent, unexpected to observe that this concentration of Cd^{2+} did not completely abolish the peak current. This might be due to the flow artifacts when Cd^{2+} was superfused over cells, or that DR neurones are somehow less sensitive to the drug, when compared to the chick sensory neurones

and rat forebrain neurones where the block was fast and complete (Swandulla & Armstrong, 1988).

Figure 3.5 shows the current elicited with a voltage step from V_H -100 mV to -10 mV for 150 ms. The current peaked in control solution at 1.82±0.08 nA and following an application of 0.1 mM Cd^{2+} it peaked at 0.35±0.04 nA, as illustrated in Figure 3.4.

When Cd^{2+} was applied in the same fashion, but in a higher concentration (1 mM) the inhibition was almost complete, and averaged 98.3±1.2%, n= 3. Following wash-out only partial recovery was achieved.

Effect of temperature on calcium currents

As some groups have already demonstrated, Ca current amplitude and kinetics are temperature-dependent (Byerly et al. 1984; VanLunteren et al. 1993). VanLunteren et al. (1993) reported that activation and inactivation of Ca currents were more rapid at higher temperatures. In bullfrog sympathetic neurones, they found that the voltage required to elicit the peak Ca current shifts ~5 mV to the left, when the temperature is increased from 20° to 30°C. A very detailed study of the effect of temperature on the HVA Ca currents in DR neurones has been performed by McAllister-Williams (1992).

In McAllister-Williams experiments, it was observed that in DR neurones the HVA current was less stable at higher temperatures and, as a consequence, long term recordings were not possible. For that reason, in the present experiments DR neurones were held at 20° C and then stepped to the desired temperatures for short periods. An

accurate temperature controller was used that allowed relatively fast temperature changes. Usually, it took a few more minutes to lower the temperature than to increase it. Because of the "run-down" of Ca current, it was difficult to obtain precise and comparable data for analysis. Therefore, when the temperature was returned to 20° C from a test temperature, a 10% change from the previous control current amplitude at 20° C was taken as acceptable. When the current stabilised at the new control level at 20° C a new temperature step was instigated.

In Figure 3.6, the time course of changes in the peak Ca current amplitude is shown, in a typical cell where the temperature was stepped in a range from 15° C to 30° C. The peak current stabilised at 20° C and the temperature increased to 25° C. The size of the current increased dramatically to 168% of the control peak current at 20° C. The temperature was then returned first to 20° C and then to 15° C. At 15° C, the current peaked at 81% of the control amplitude at 20° C. The current was stabilised at 20° C again and the temperature stepped to 30° C, where, as expected, the current was significantly larger, and peaked at 191% of the control peak current. "Run-down" process was much faster at higher temperatures. At 25° C, the current decreased 11% over a 3 min period. At 30° C, the decrease after 3 min was 14%, while only 3.6% over 3 min when holding the cell at 15° C. In all cells tested, the result followed that pattern (n= 4). The data show that Ca channels functioning is strongly dependent on temperature and confirm reports from other laboratories (Byerly et al. 1984; VanLunteren et al. 1993).

In Figure 3.7 Ca current amplitude at different temperatures is plotted against voltage. At all temperatures tested (15°, 20° and 25° C), Ca current was voltage-dependent and peaked at around -10 mV. Furthermore, the biggest difference in the current amplitudes was observed at around the peak amplitudes. At more depolarising test potentials, the size of the current was less temperature-dependent. The reversal potential was 50 mV at all temperatures.

The data was averaged from 4 neurones. Only the cells in which a complete range of temperatures was tested (from 15° to 25°C) are included in the analysis. In 2/4 recordings, the Ca current at 25°C peaked at slightly more negative potentials, close to -20 mV. Other workers, McAllister-Williams (1992) and VanLunteren et al. (1993), have also observed this shift in the peak potential to the left. The present data could be similar, but the potentials were stepped at 10 mV intervals, and could only be approximate for the peak current obtained.

A change in temperature, in most cells, affected both, transient and slowly decaying, components of the peak Ca current (Figure 3.8). In majority of neurones both components were potentiated at the higher temperature, that is best seen when the current obtained at 20°C is subtracted from that obtained at 25°C, see inset in (A). In other cells, only transient, possibly N-type current was modulated, as in (B).

Modulation of calcium currents by 5-HT_{1A} agonist

Inhibition of calcium currents by neurotransmitters has been described in many cell types, such as chick sensory neurones (Dunlap & Fischbach, 1978), cerebellar granule neurones (Pearson et al. 1993), chromaffin cells (Gandía et al. 1993), frog sympathetic neurones (Elmslie et al. 1992; Boland & Bean, 1993; Elmslie et al. 1993), Helix aspersa (Hill-Venning & Cottrell, 1992), rat dorsal root ganglion (Bean, 1989a; Dolphin et al. 1989; Menon-Johansson et al. 1993), rat hypothalamic neurones (Zeilhofer et al. 1993), guinea-pig submucosal neurones (Surprenant et al. 1990) and DR neurones (Penington et al. 1991).

In DR neurones, Penington et al. (1991) and McAllister-Williams (1992) reported that 8-OH DPAT, a potent 5-HT_{1A} agonist, partially inhibited the HVA Ca current.

The action was blocked by the putative 5-HT_{1A} antagonist 1-(2-methoxyphenyl)-4-[4-(2-phthalimmido)butyl] piperazine (NAN-190).

In Figure 3.9 the peak Ca current is plotted against time. After establishing the whole-cell mode, the peak Ca current initially increased and then stabilised. A second, small glass pipette with a broken tip was lowered into the standard external solution and 8-OH DPAT perfused from the pipette into the vicinity of the cell. The bath flow was stopped and known final concentration of the drug was achieved. 8-OH DPAT was used rather than 5-HT since it is not involved in the process of neuronal uptake and is more stable and suitable for testing at different temperatures.

Invariably, Ca current was only partially blocked with a supramaximal dose of 8-OH DPAT (50 μM), on average, by 34.3±3.8%. This is a smaller percentage of inhibition than reported for 5-HT by Penington et al. (1991). One explanation could be that the concentration of 8-OH DPAT was not optimal or that the cell did not see the full dose of the drug. Another, more likely, possibility is that while 5-HT is a full agonist on 5-HT_{1A} receptors, 8-OH DPAT is, arguably, only a partial agonist (Sprouse & Aghajanian, 1988; Sprouse et al. 1993). In hippocampal neurones, the lower potency of 8-OH DPAT, compared to 5-HT, was attributed to lower reserves of 5-HT_{1A} receptors, to explain its action as a partial agonist at 5-HT_{1A} receptors (Andrade & Nicoll, 1987; Sprouse & Aghajanian, 1988; Sprouse et al. 1993).

The inhibition of the current was variable across cells. The reason might be that the perfusion of the cells by 8-OH DPAT was not adequate or the wash-out of intracellular components occurred. Another possibility was a difference in the cell population. This is probably unlikely, because, Lawrence et al. (1989) showed that ~90% of DR neurones contain 5-HT_{1A} receptors, and 8-OH DPAT had no effect in ~8% of the cells (4 out of 51). Moreover, DR neurones have a very small variation in the cell diameter, so differences in responses can not be attributed to a different size in Ca currents that appear to depend on cell diameter, as proposed by others (Scroggs & Fox, 1992b).

The current recovered slowly after removal of 8-OH DPAT from the standard external solution. In a minority of DR neurones, as in the cell shown, the post-application Ca current peaked at a higher amplitude. Similar "overrecovery" has also been observed in other neurones (Boland & Bean, 1993).

Control Ca current at various potentials is compared with Ca current elicited in the presence of 8-OH DPAT, see Figure 3.10. The currents were evoked from V_H -100 mV to the indicated test potentials. The presence of 8-OH DPAT did not change the voltage-dependency of Ca channels and the potential at which the Ca current peaked. Kasai and Aosaki (1989) showed that 2-chloroadenosine (2-CA) inhibited Ca channel current in chick sensory neurones most prominently at the onset of depolarisation, and the inhibition slowly reached a steady level. This group observed no depression of the current at potentials more positive than 30 mV. Surprenant et al. (1990) reported no effect of the action of noradrenaline in guinea-pig submucosal neurones on membrane potential. In DR cells, block of Ca current started at depolarising potential of -50 mV, and the maximal inhibition was seen at -10 mV. At negative test potentials, -40 mV, -30 mV, -20 mV and -10 mV, the current was blocked by 15, 23, 29 and 34%, respectively. At more positive potentials, the inhibition was less and at 0, 10 and 30 mV it was 25, 14 and 4%, respectively. At around 40 mV, no block was observed. The reversal potential was not affected, and occurred at 50 mV. The inhibition at more positive potentials was not examined, but others (Bean, 1989a; Boland & Bean, 1993) showed that the inhibition by transmitter ligands was much smaller at larger depolarisations. In bullfrog sympathetic ganglion neurones, LHRH inhibited Ca current evoked with a voltage step to -10 mV by 28%, whereas the current elicited with a step to 140 mV was blocked by only 11% (Boland & Bean, 1993).

In Figure 3.11 the peak current traces of control Ca current, during an application of 50 μM 8-OH DPAT and recovery are shown. 8-OH DPAT inhibited the peak Ca

current and prolonged the activation time (A). In the inset, the inhibited part of the current is shown following subtraction. Both, a fast transient and slowly decaying components were inhibited by 8-OH DPAT. Note the "overrecovery" that occurred after the removal of the drug. In the same cell the full process of recovery after wash-out of the drug is shown in (B) by superimposing repetitive current traces. Recovery of both components was fast and simultaneous.

A number of groups (Artalejo et al. 1992b; Elmslie, 1992; Kasai, 1992; Boland & Bean, 1993; Elmslie et al. 1993; Gandía et al. 1993; Golard & Siegelbaum, 1993; Zeilhofer et al. 1993) have shown that a prepulse can partially relieve the inhibition of Ca current caused by an agonist. As described earlier, in DR neurones a prepulse did not change the size of the peak current in the absence of 5-HT_{1A} receptor stimulation. During the presence of 8-OH DPAT, a prepulse to 40 mV from V_H -100 mV, 10 ms before a test pulse was applied and shown to substantially relieve the inhibition of Ca current, on average, by 61%, see Figure 3.12.

The peak current, elicited in the presence of 8-OH DPAT, was blocked by Cd^{2+}, see Figure 3.13.

Modulation of calcium currents by GTP-γ-S

In many cell types it has been reported that inclusion of GTP-γ-S in a pipette modulate Ca current (Dolphin et al. 1989; Kasai & Aosaki, 1989; Elmslie, 1992; Golard & Siegelbaum, 1993; Netzer et al. 1994) and intracellular dialysis with GTP-γ-S, mimics the effect of extracellularly applied ligand, for review see Dolphin (1991a). This suggests that G-proteins are involved in the coupling of activated receptors to the Ca channels.

GTP-γ-S (200 μM), a non-hydrolysable analogue of GTP that irreversibly activates G-proteins, was included in a recording pipette. Ca current, elicited with standard voltage steps from V_H -100 mV to -10 mV, at 20°C, peaked at 1.02±0.04 nA, significantly lower than in control DR cells (1.82±0.08 nA, $p<0.001$), see Figure 3.14. Another difference was that the peak current reached its maximal amplitude some what later than in control experiments. With GTP-γ-S in the recording pipette, the maximal peak current was seen in 9-15 min (mean= 11 min, n= 16) after obtaining a whole-cell mode, compared to 7.6 min in control cells. At 20°C, "run-down" of the current was smaller and the peak current decreased by 5.6±2.3%, 30 min later.

The assumption was that the effect of 8-OH DPAT on Ca channel current was the result of G-protein activation. Therefore, it was to expect that when the G-protein was already fully and irreversibly activated by GTP-γ-S, no further change either in the current amplitude or activation kinetics would occur with the addition of 8-OH DPAT. The current recorded in addition of 8-OH DPAT (50 μM), peaked at 0.98±0.03 nA, n= 4, thus not significantly different from the result obtained in GTP-γ-S treated cells. The second application of 8-OH DPAT, onto GTP-γ-S-dialysed DR neurones, also produced no significant effect. Therefore, it is difficult to relate the suggestion made by Elmslie (1992), that the action of GTP-γ-S is gradually lost over time, as in sympathetic ganglion neurones. He also observed that LHRH and NE reversibly inhibited Ca currents, even in the presence of the GTP analogue.

From times (1) and (2) in Figure 3.14, the peak Ca currents are shown as traces in the inset. The currents were obtained in the presence of GTP-γ-S (200 μM) and GTP-γ-S + 8-OH DPAT (50 μM). GTP-γ-S inhibited the peak Ca current, and the other striking effect produced was the slowing of the activation kinetics. At the end of the test pulse (150 ms), the current was still not fully activated in most neurones, and no inactivation of the current was observed. 8-OH DPAT had no significant effect in the presence of GTP-γ-S. In experiments with GTP-γ-S, the best fit was obtained by a double exponential function, as in 8-OH DPAT cells. In control experiments, the activation followed a single exponential function.

It was interesting to test whether the HVA Ca current in the presence of GTP-γ-S is also temperature dependent. Figure 3.15 shows the effect of temperature on a GTP-γ-S dialysed cell. At 20°C, the current peaked at 1.02±0.04 nA. When the temperature was stepped to 25°C the peak current was dramatically enhanced to 1.68±0.05 nA, $p<0.001$. This effect of temperature was completely reversible.

In Figure 3.16, two superimposed peak Ca currents are shown as traces at 20°C and 25°C, respectively. At 25°C, the peak current was larger than at 20°C, and the current was fully activated during the test pulse.

Several groups of researchers have shown that the application of a brief, depolarising prepulse, shortly before the test pulse could partially reverse the effect of G-protein activation on Ca currents (Penington et al. 1991; Gandía et al. 1993; Golard & Siegelbaum, 1993). For example, Golard and Siegelbaum (1993) showed a partial recovery of the inhibition of Ca current by GTP-γ-S, following an application of a 15 ms long prepulse from V_H -80 mV to 100 mV. In the presence of GDP-β-S, the prepulse had no effect on Ca currents.

In DR neurones dialysed with GTP-γ-S, a prepulse to 40 mV for 100 ms, 20 ms prior to the test pulse, potentiated Ca current and the peak was, on average, at 1.11±0.02 nA, see Figure 3.17. So, the prepulse partially relieved the inhibition of the peak Ca current at 20°C, and also restored the activation kinetics. At 25°C, the effect of the prepulse was similar, see Figure 3.18.

Therefore, the result obtained in the presence of GTP-γ-S is similar to the effect of the prepulse application on the peak current in cells bathed in 8-OH DPAT.

Current-voltage relation of Ca current amplitudes at 20° and 25°C is shown in Figure 3.19. The current activated around potentials positive to -40 mV, and, at both temperatures, peaked at around -10 mV. Reversal potential was at 50 mV, as in control cells. As explained earlier, the current amplitude was larger at 25°C and the biggest difference in the current amplitude occurred at the potential that evoked peak

amplitudes. At potentials more positive than 10 mV, there was no significant difference in the current size at the two temperatures.

As a control for the action of GTP-γ-S, 2 mM of GDP-β-S was added to the standard internal solution. Peak current amplitudes are plotted against time and shown in Figure 3.20. The current peaked, on average, at 1.89±0.04 nA, n= 4. This was not significantly different from the peak Ca current in control experiments, 1.82±0.04 nA. "Run-down" of the peak Ca current was also similar to control cells and 20 min after obtaining the maximal peak amplitude, it measured 14±2.1%.

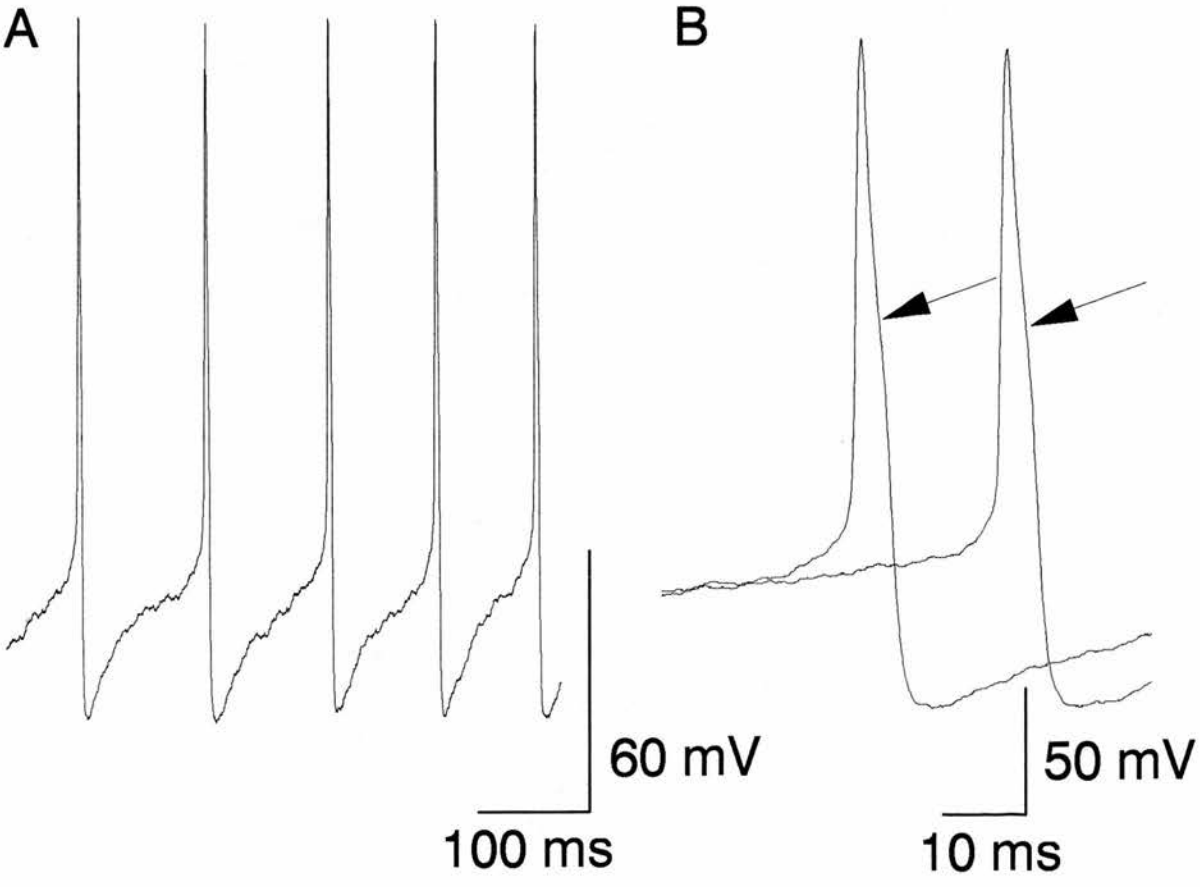

Figure 3.1: Action potentials recorded from acutely dissociated DR neurone. (A) Whole-cell current-clamp recording showed many neurones to fire spontaneous action potentials. The potentials measured between 70-100 mV and could also be evoked by depolarising pulses positive to -60 mV (A). In (B) the same potentials are shown on an expanded time scale. Each of them consisted of an initial depolarisation, a small shoulder during repolarisation (arrow) and a hyperpolarisation. K^+ gluconate (120 mM) in internal solution and standard external solution with no TTX were used. Cell 1906922.

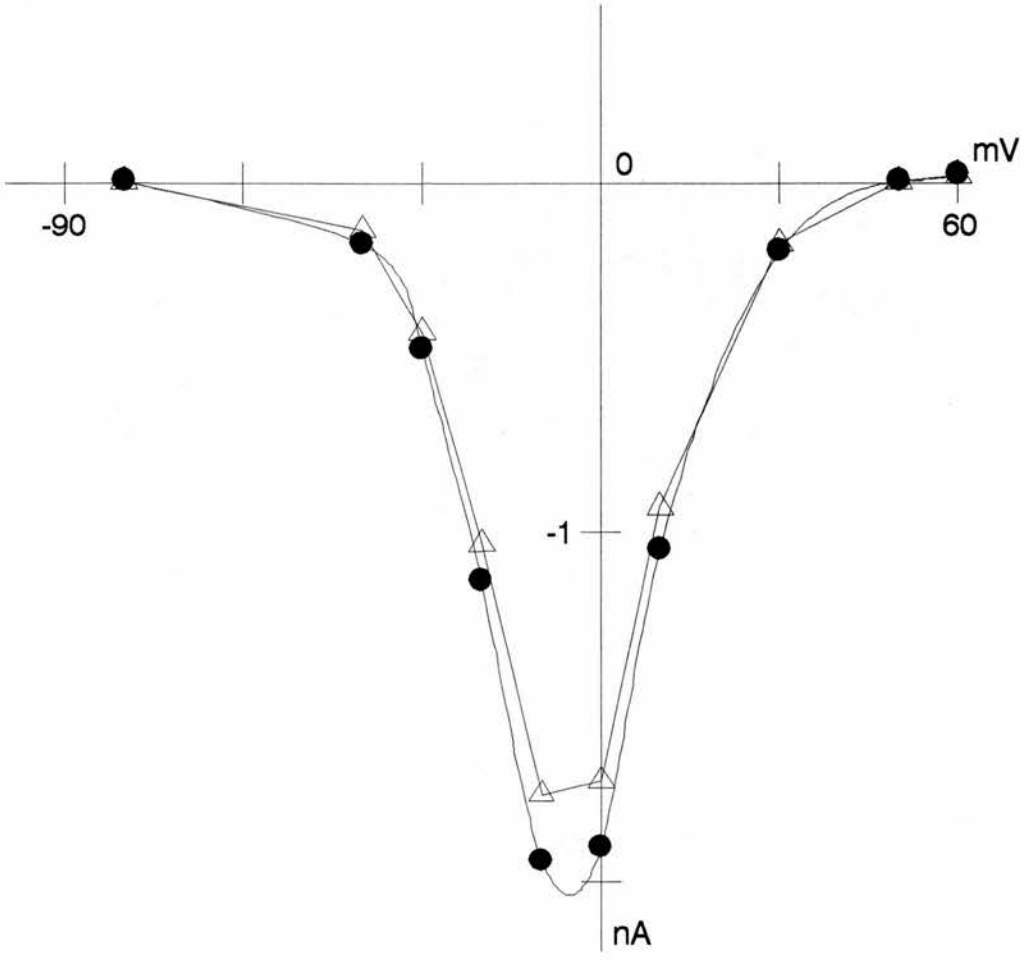

Figure 3.2: Current-voltage relationship of calcium currents recorded in a typical DR neurone at 20° C. Ca currents were elicited by voltage steps from a holding potential of -100 mV to a series of depolarised test potentials for 150 ms, as indicated. The relation between the test potentials and transient (●) and slowly decaying (△) Ca current components is shown. At test potentials positive to -40 mV, the current increased and peaked at around -10 mV. The reversal potential was at 50 mV. Note that strong inactivation of the current occurred only at the peak amplitude. The curve was fitted using a cubic spline routine. Standard internal and external solutions were used. Cell 1401933.

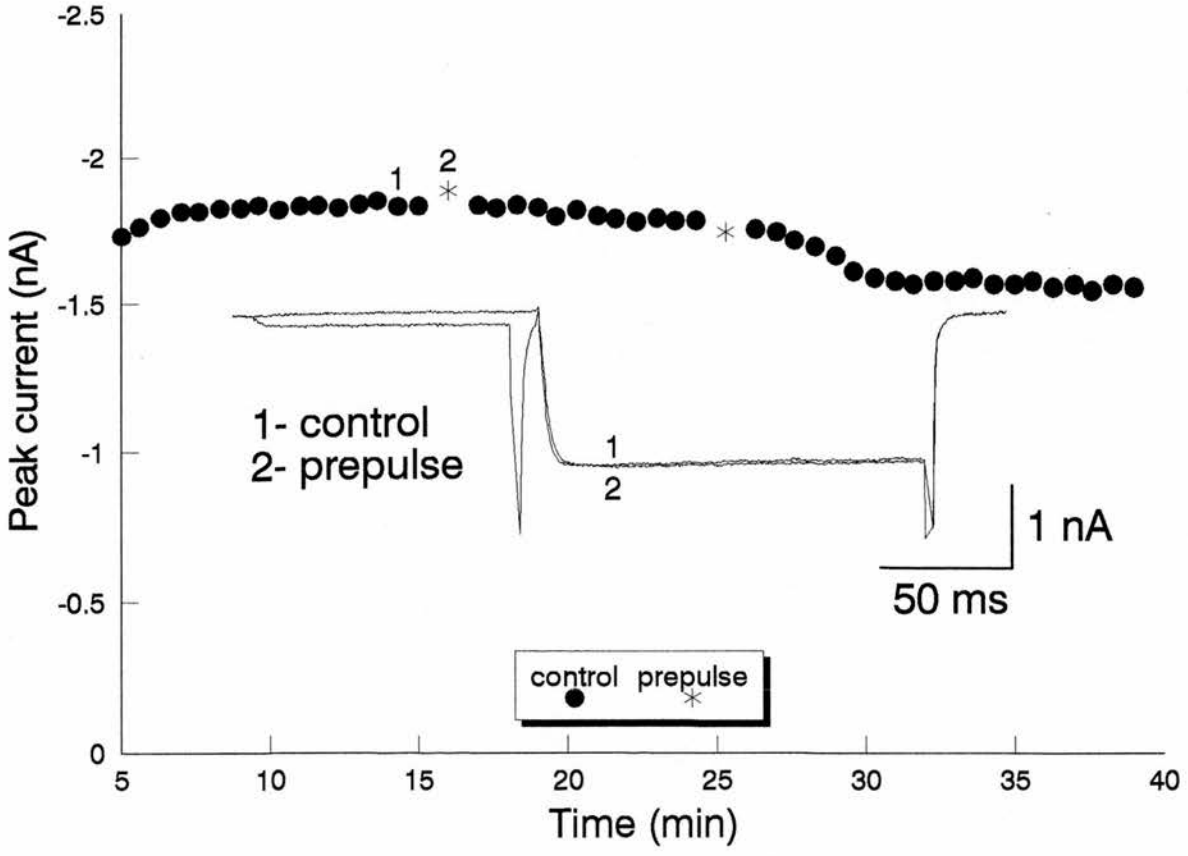

Figure 3.3: "Run-down" of the peak calcium current with time and the effect of prepulse. Peak amplitude (●) of the HVA Ca current evoked by a voltage step from V_H -100 mV to -10 mV at a frequency of 0.05 Hz and at 20°C exactly, is plotted against time. Maximal amplitude of the peak current was obtained, on average, in 7.6 min. The peak Ca current stabilised, on average, at 1.82 nA and 30 min later it was 17% smaller, n= 34. Every second peak is plotted. A prepulse (✱) applied intermittently 10 ms prior to the test pulse, from V_H -100 mV to 40 mV and 100 ms in duration, produced no significant change in the current amplitude, that peaked, on average, at 1.86 nA, n= 16. The inset shows superimposed Ca current traces in the presence and in the absence of a prepulse. Currents are leak and capacity subtracted. Standard internal and external solutions were used. Cell 0902932.

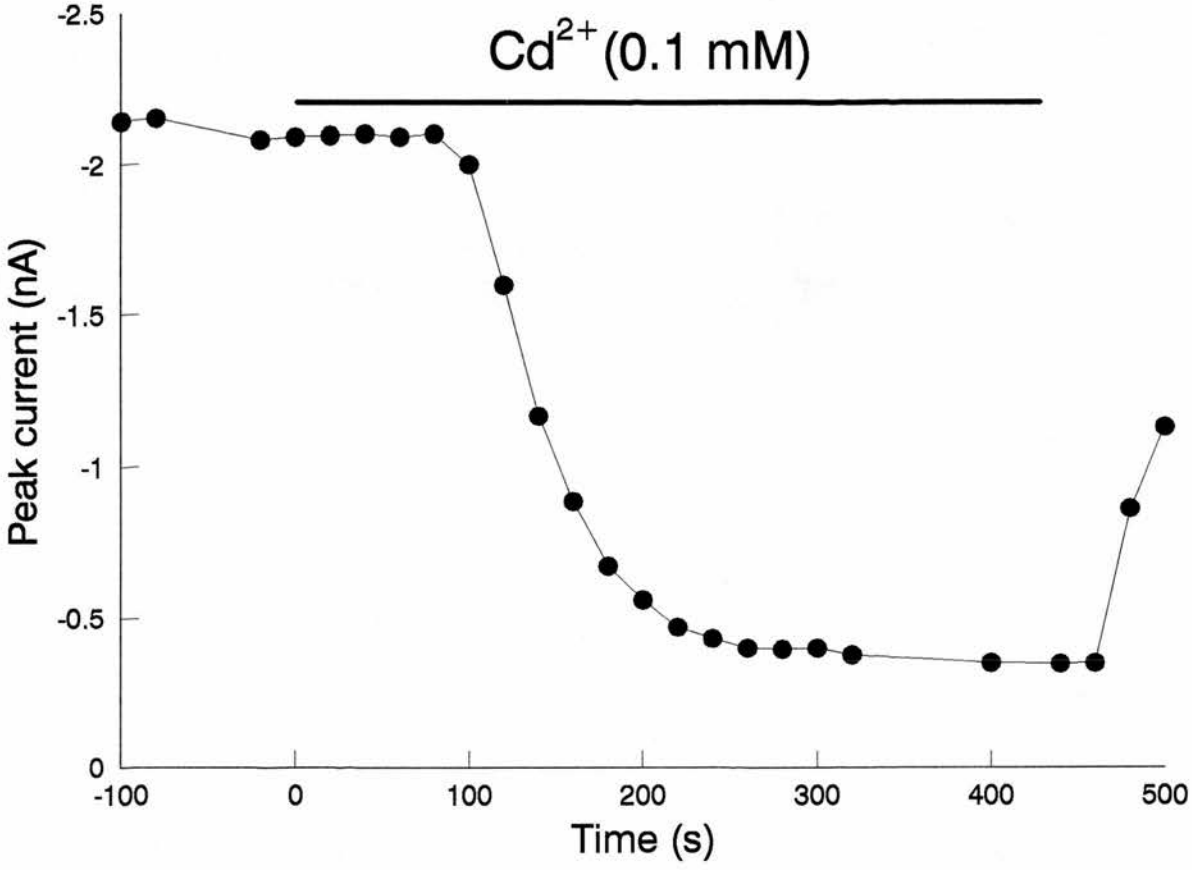

Figure 3.4: Time course of block of the peak calcium current by cadmium. Peak calcium current (●) evoked by a standard voltage pulse is plotted against time. At indicated time "0", the peak current stabilised and 0.1 mM of Cd^{2+} was applied externally for 7 min (horizontal bar) to the cell. The drug reduced the peak Ca current on average by 81%, n= 3. Cadmium was perfused by a broken glass electrode with 3-5 μm tip diameter into the vicinity of the neurone. Standard internal and external solutions were used. Currents are leak and capacity subtracted. Cell 2001932.

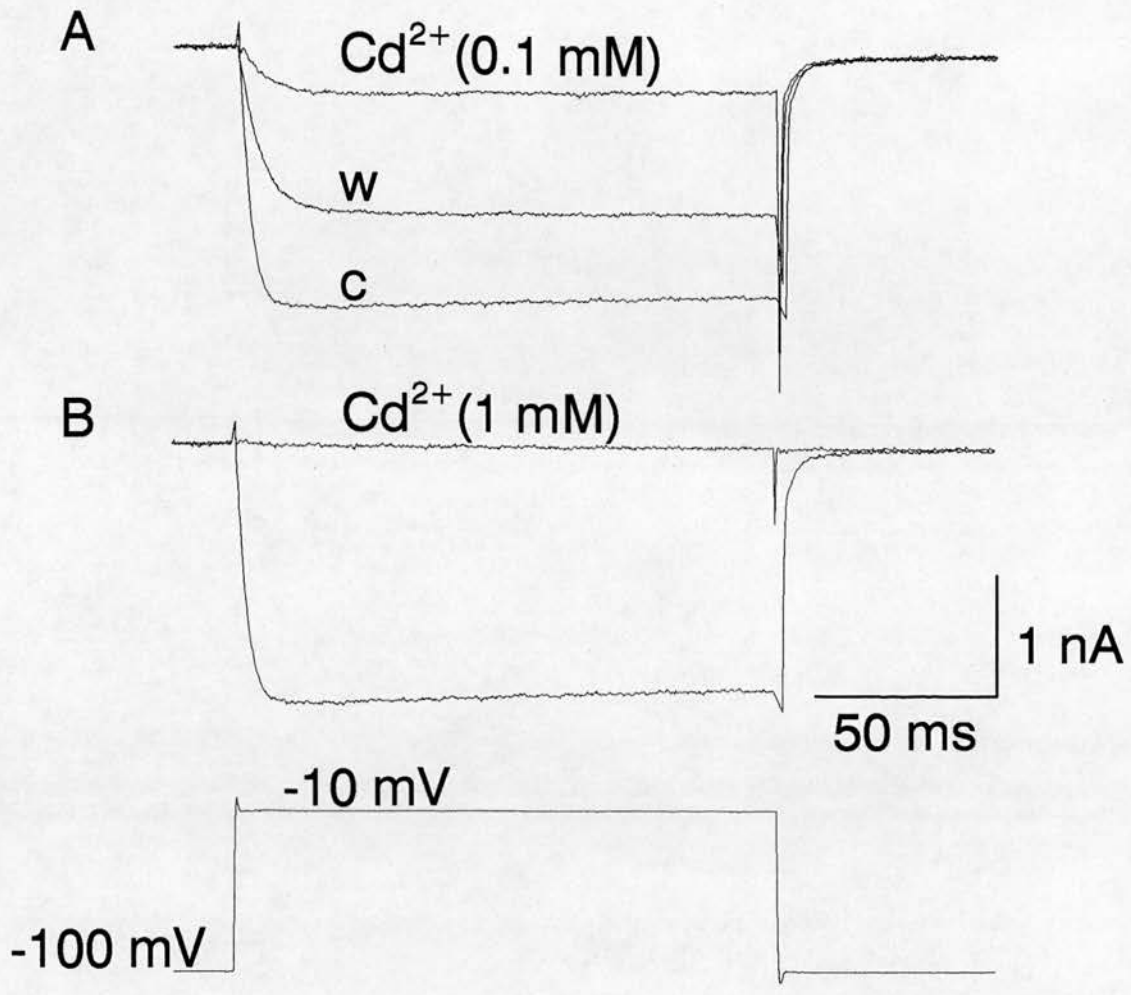

Figure 3.5: Inhibition of the peak calcium current by various concentrations of cadmium. Cd^{2+} was applied externally to the vicinity of the neurone and superimposed peak Ca current traces are shown. In the concentration of 0.1 mM, Cd^{2+} reduced the peak Ca current by 81%, n= 3 (A), and 1 mM almost completely abolished the current by 98%, n= 3 (B). The inhibition was partially reversible, following wash-out of Cd^{2+} (W). Standard internal and external solutions were used. The peak Ca currents were evoked with standard voltage pulses and leak and capacity subtracted. Voltage trace is shown in the lower panel. Cell 2001933.

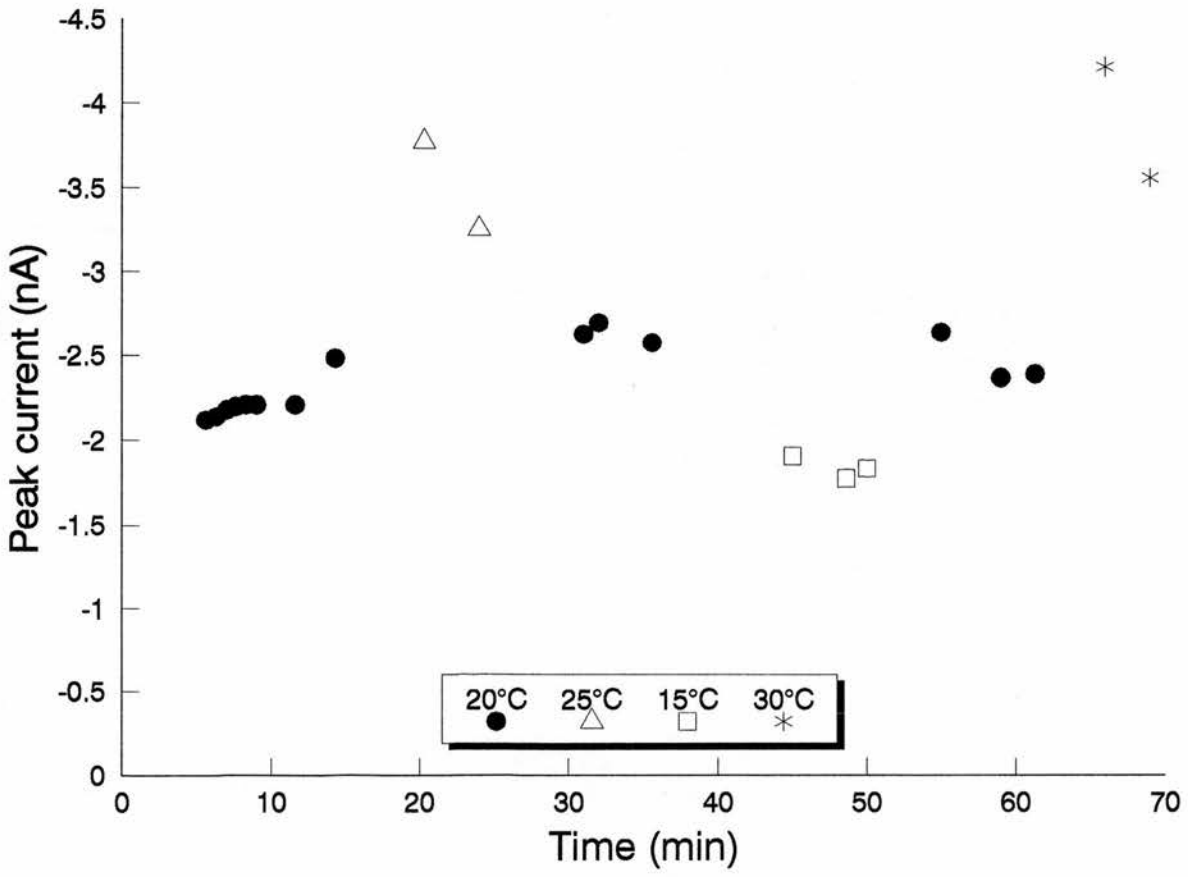

Figure 3.6: Effect of temperature on the peak calcium current. Temperature-dependency of the peak Ca current in DR neurones is shown over a period of time. At 20°C (●) the peak current stabilised and with a temperature step to 25°C (△) increased to 168% of control. The current then equilibrated at 20°C and the temperature was lowered to 15°C. At 15°C (❑) the current peaked at 81% of the control amplitude observed at 20°C. The temperature was returned to 20°C and then jumped to 30°C (✱), where the current peaked to 191% compared to the control amplitude, n= 4. "Run-down" was faster at higher temperatures, see text. Standard voltage protocol was employed and standard internal and external solutions were used. Cell 2201931.

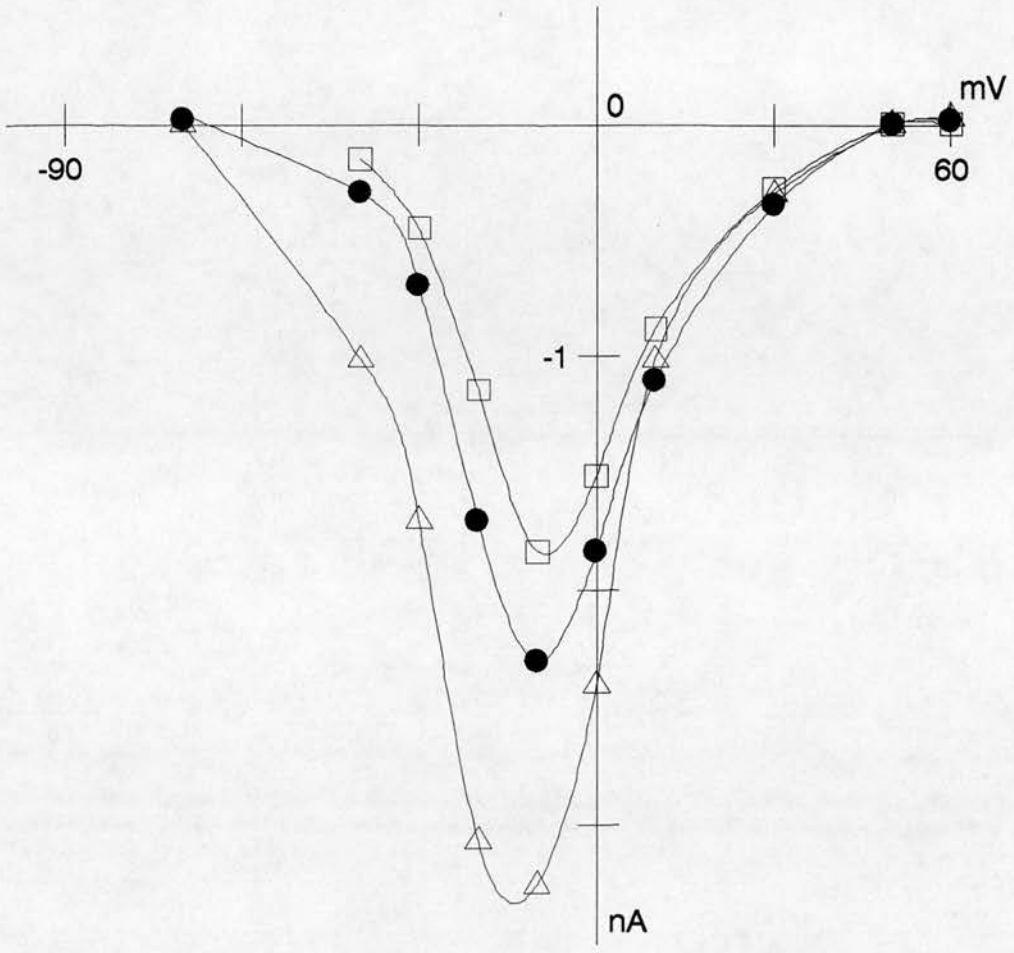

Figure 3.7: Current-voltage relationship of calcium currents recorded at various temperatures. Calcium currents were elicited with voltage steps from a holding potential of -100 mV to indicated potentials in a representative DR neurone. At all tested temperatures, that is at 15°C (❑), 20°C (●) and 25°C (△) the current was voltage-dependent, peaked at around -10 mV and reversed at 50 mV. The current amplitudes were best differentiated at the peak potential. Note that at more depolarising potentials the currents were less temperature-dependent. The curve was fitted by a cubic spline routine. Currents are leak and capacity subtracted. Standard internal and external solutions were used. Cell 2101931.

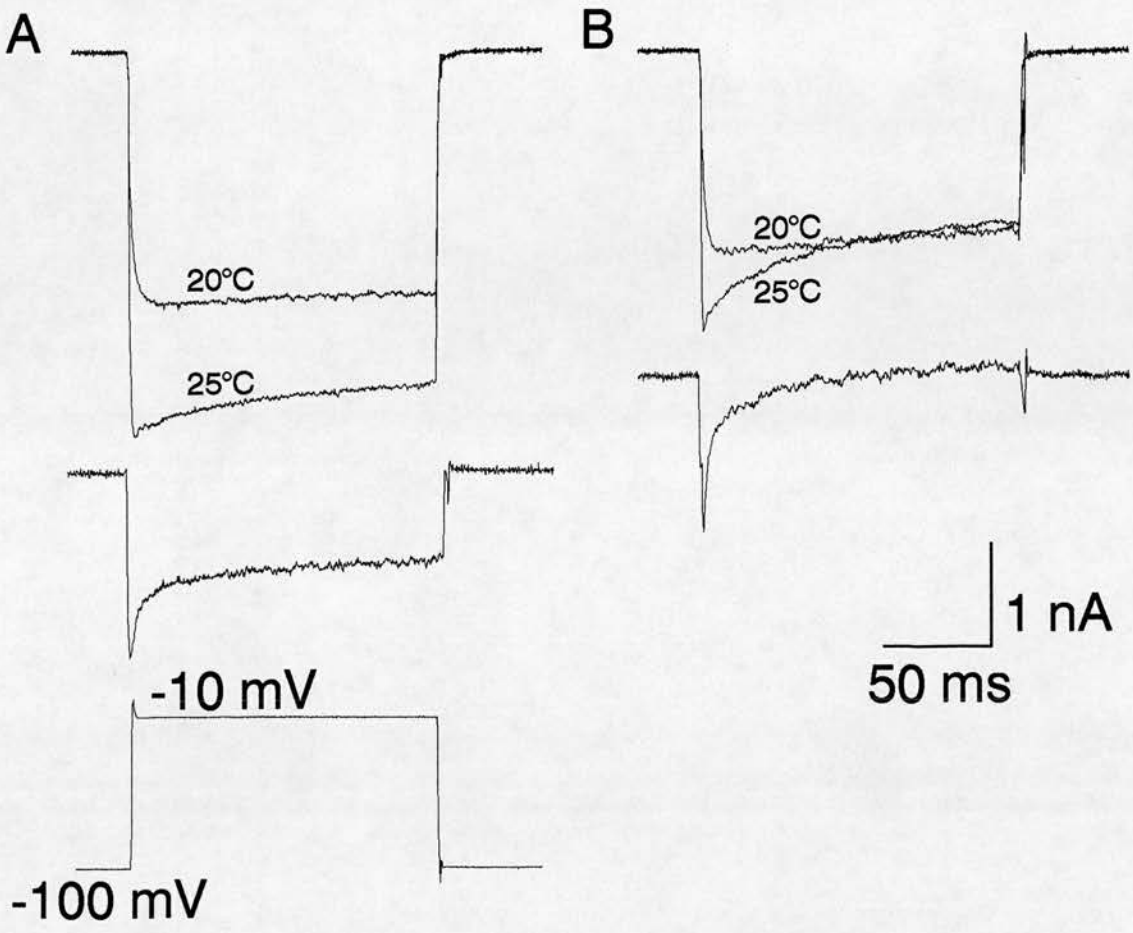

Figure 3.8: Effect of temperature on the peak calcium currents at 20° and 25°C. Ca currents elicited with a standard voltage steps peaked, on average, at 1.82 nA (n= 34) and 3.05 nA (n= 4), at 20° and 25°C, respectively. In most neurones both transient and slowly decaying components of the peak current were affected by an increase in temperature (A), although in minority of DR neurones only transient part of the current was potentiated (B). The insets show Ca current when the current obtained at 20°C was subtracted from that obtained at 25°C. Currents are leak and capacity subtracted. Standard internal and external solutions were used. Cells 2701932 (A) and 1102931 (B).

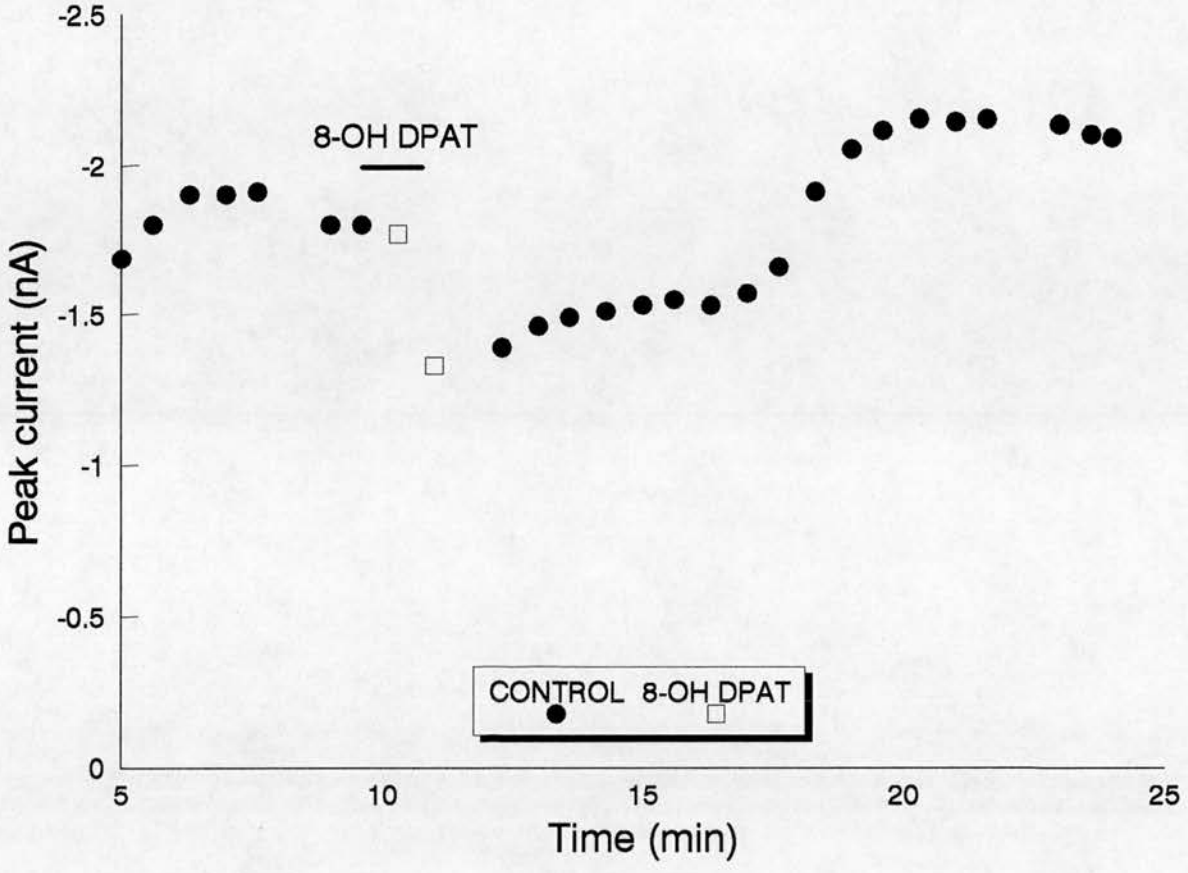

Figure 3.9: Time course of 8-OH DPAT-induced inhibition of the peak calcium current. Peak Ca current (●) was evoked using standard voltage protocol in a typical DR neurone. The current stabilised and 8-OH DPAT (50 μM, ❑) was applied (horizontal bar) externally via a broken tip capillary glass close to the neurone. The compound led to a large (34.3%) but incomplete reduction of the peak current, n= 22. The inhibition was reversible and following wash-out of 8-OH DPAT the peak Ca current fully recovered. In a number of cells an "overrecovery" was observed, see text. Currents are leak and capacity subtracted. Standard internal and external solutions were used. Cell 2204931.

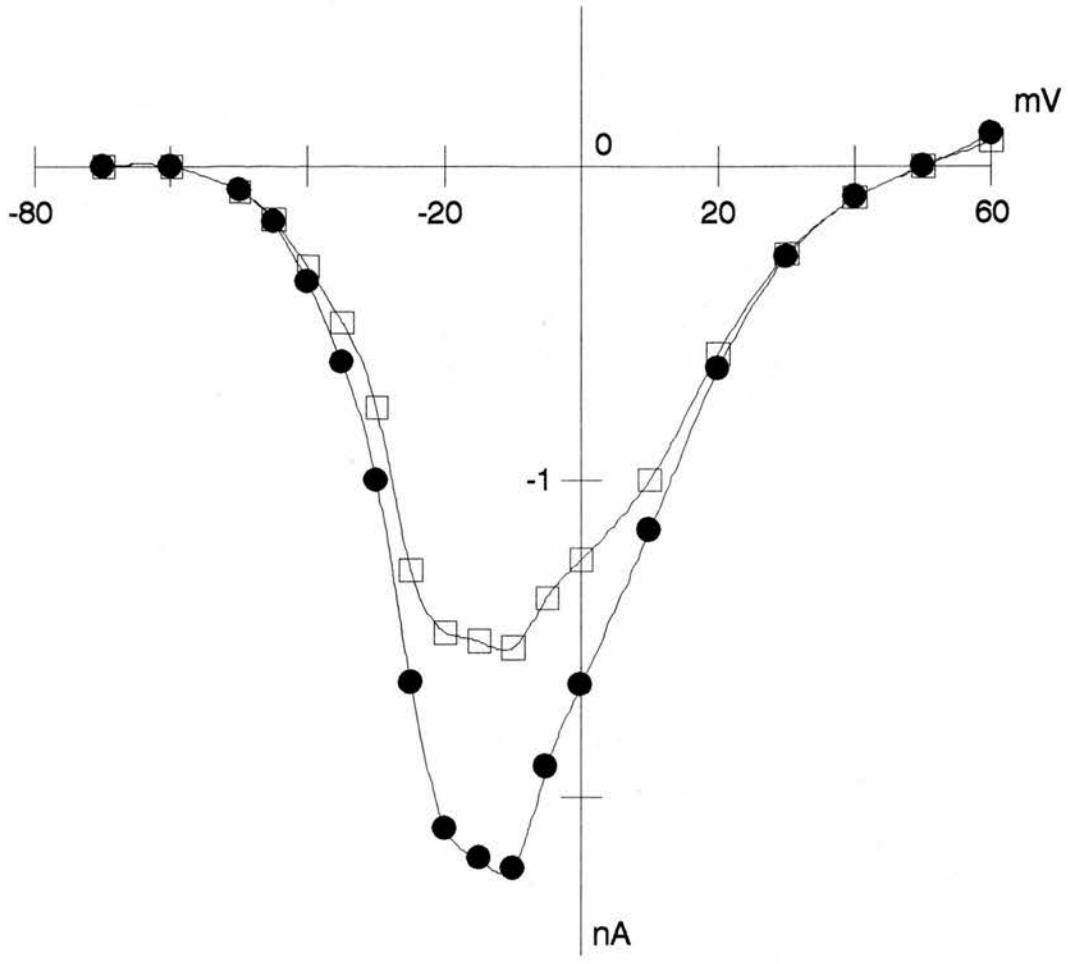

Figure 3.10: Current-voltage relationship of calcium currents recorded in the presence and in the absence of 8-OH DPAT. From a holding potential of -100 mV jumps to the indicated test potentials were used to evoke and compare control Ca currents (●) with the currents elicited in the same fashion, but in the presence of 8-OH DPAT (50 μM, ❑). Currents evoked in both conditions were voltage-dependent. The inhibition of Ca currents started at potentials at around -50 mV, with the maximal block at -10 mV. The inhibition was weaker at positive test potentials and the current reversed at around 50 mV, as in control situation. The curve was fitted using a cubic spline routine. Currents are leak and capacity subtracted. Standard internal and external solutions were used. Cell 2403931.

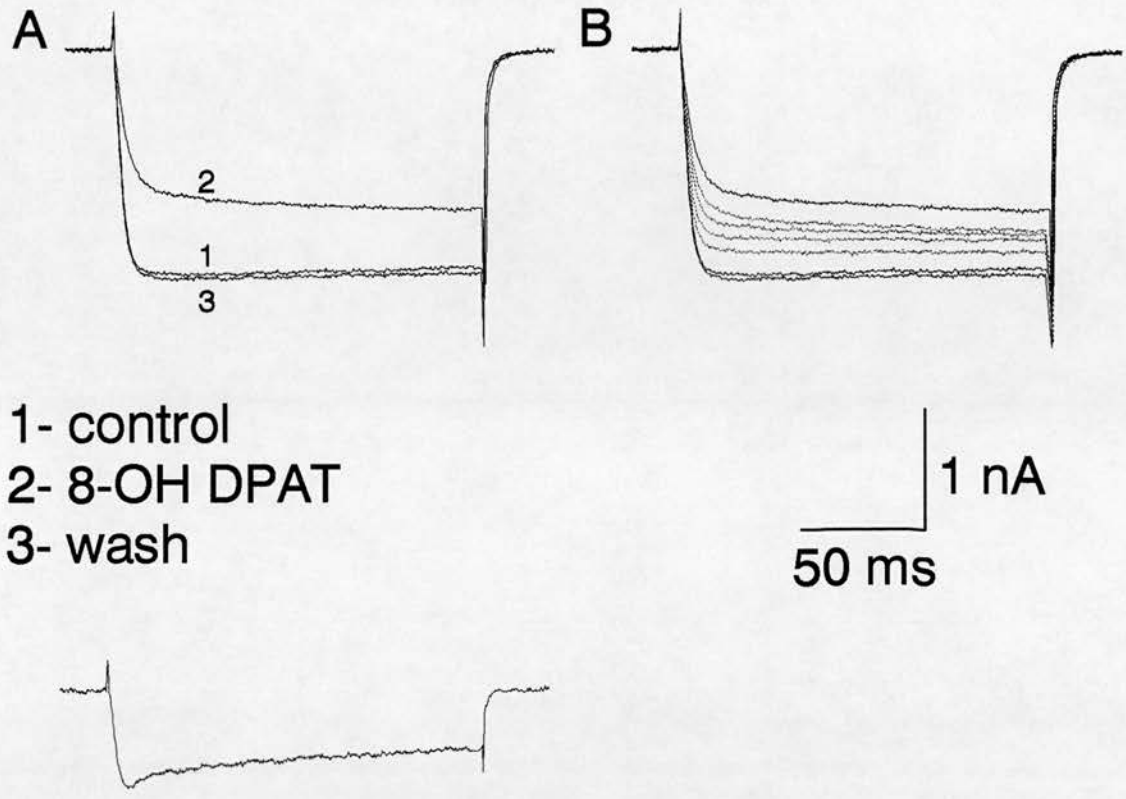

Figure 3.11: Inhibition of the peak calcium current by 8-OH DPAT. Control Ca current (1) peaked, on average, at 1.82 nA (n= 34) and following the application of 8-OH DPAT (50 μM, 2) significantly decreased and peaked, on average, at 1.21 nA (n= 22, $p< 0.001$). The 5-HT_{1A} agonist was applied into the standard extracellular solution close to the DR neurone. Following wash-out (3) of 8-OH DPAT, recovery of the current amplitude was complete (A). Inset shows the inhibited component of the current. In (B) a full process of recovery (···) following removal of 8-OH DPAT is shown by superimposing current traces. Both transient and slowly decaying components of the current recovered simultaneously. Currents are leak and capacity subtracted. Standard internal and external solutions were used. Cell 0903932.

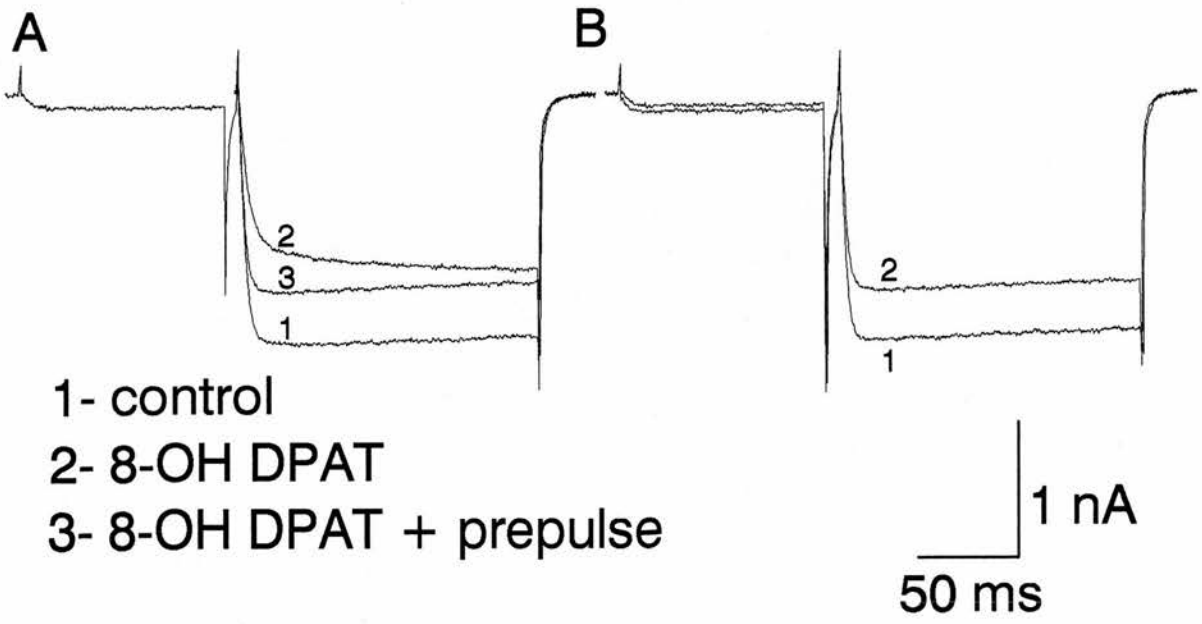

Figure 3.12: 8-OH DPAT-induced reduction of the peak calcium current is significantly relieved by a prepulse. The peak Ca current (1) was blocked by 8-OH DPAT (50 μM, 2) on average by 34%, n= 22. During continuous presence of the 5-HT_{1A} agonist in the external solution, a prepulse (3) applied to 40 mV from V_H -100 mV, 10 ms prior to the test pulse, substantially reversed the inhibition by 61%, n= 9 (A). In (B) the peak Ca current traces obtained following a prepulse application in control current (1) and in the presence of 8-OH DPAT (2) are superimposed for comparison and show a complete restoration of the activation kinetics. Currents are leak and capacity subtracted. Standard internal and external solutions were used. Cells 0903932 and 0903933.

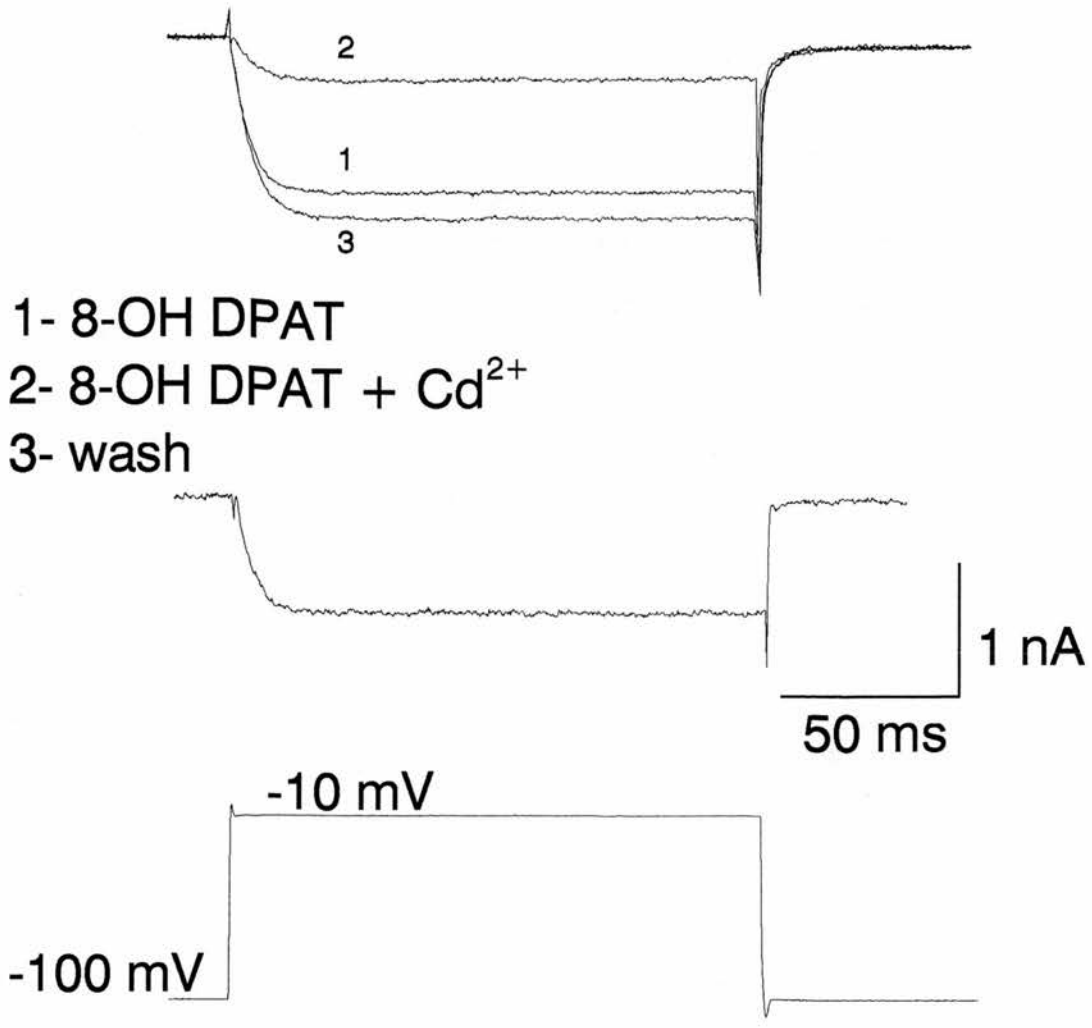

Figure 3.13: Cadmium inhibited the peak calcium current in the presence of 8-OH DPAT. 8-OH DPAT (50 μM, 1) partially blocked the peak current by 34% and in addition of Cd^{2+} (100 μM, 2) the current was reduced by 74%, n= 2. The effect of Cd^{2+} was fully reversible (3) in this DR neurone. Peak Ca currents were elicited with standard voltage pulses (lower panel). The inset shows the current that was inhibited following Cd^{2+} application. Currents are leak and capacity subtracted. Standard internal and external solutions were used. Cell 1203931.

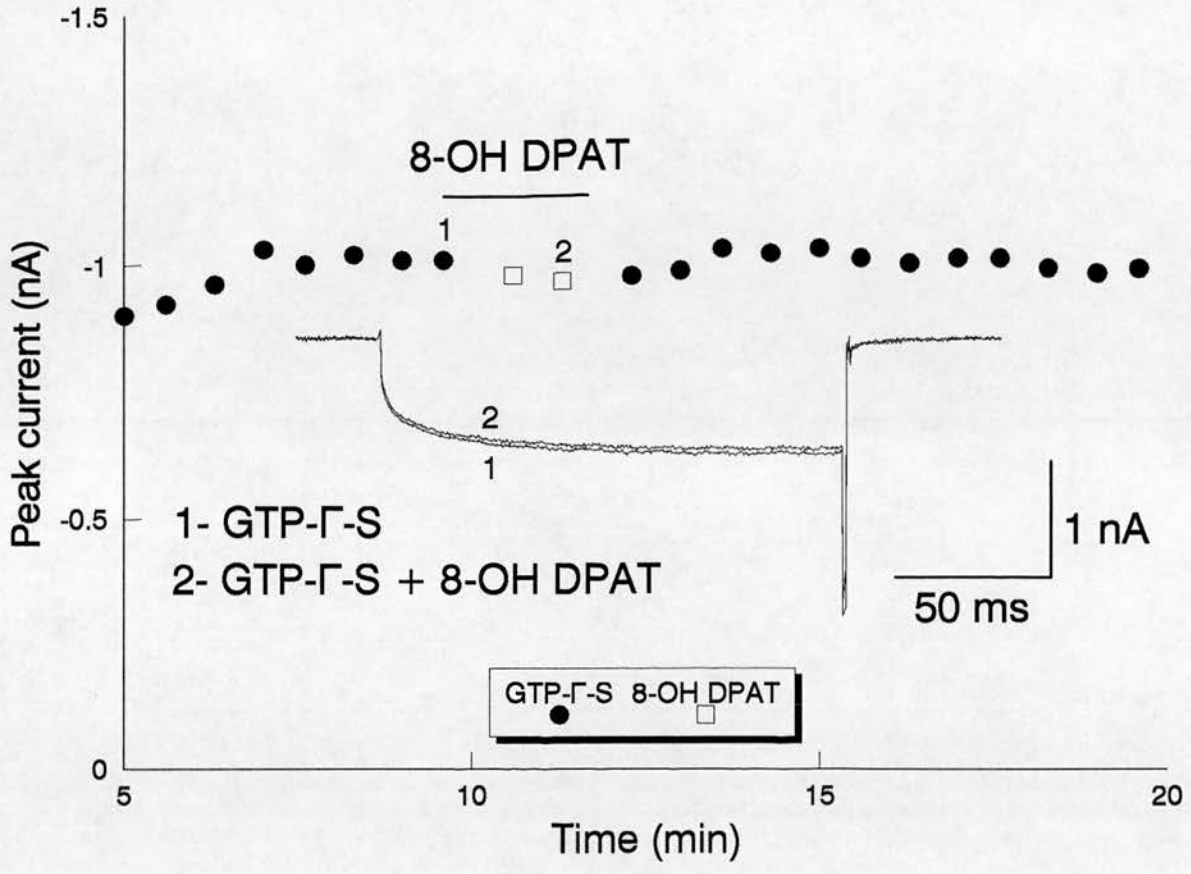

Figure 3.14: The inhibition of the peak calcium current by 8-OH DPAT is G-protein mediated. Peak Ca current amplitude (●) in a DR neurone dialysed with GTP-γ-S (200 μM) is plotted against time. At the time indicated by the horizontal bar, 8-OH DPAT (50 μM, ❑) was applied externally to the DR neurone, for 2 min. The compound had no significant effect on the size of the peak current amplitude. Inset shows two superimposed peak Ca current traces obtained in the presence of GTP-γ-S (1) and in addition of 8-OH DPAT (2). Currents are leak and capacity subtracted. Standard internal and external solutions were used. Cell 1105931.

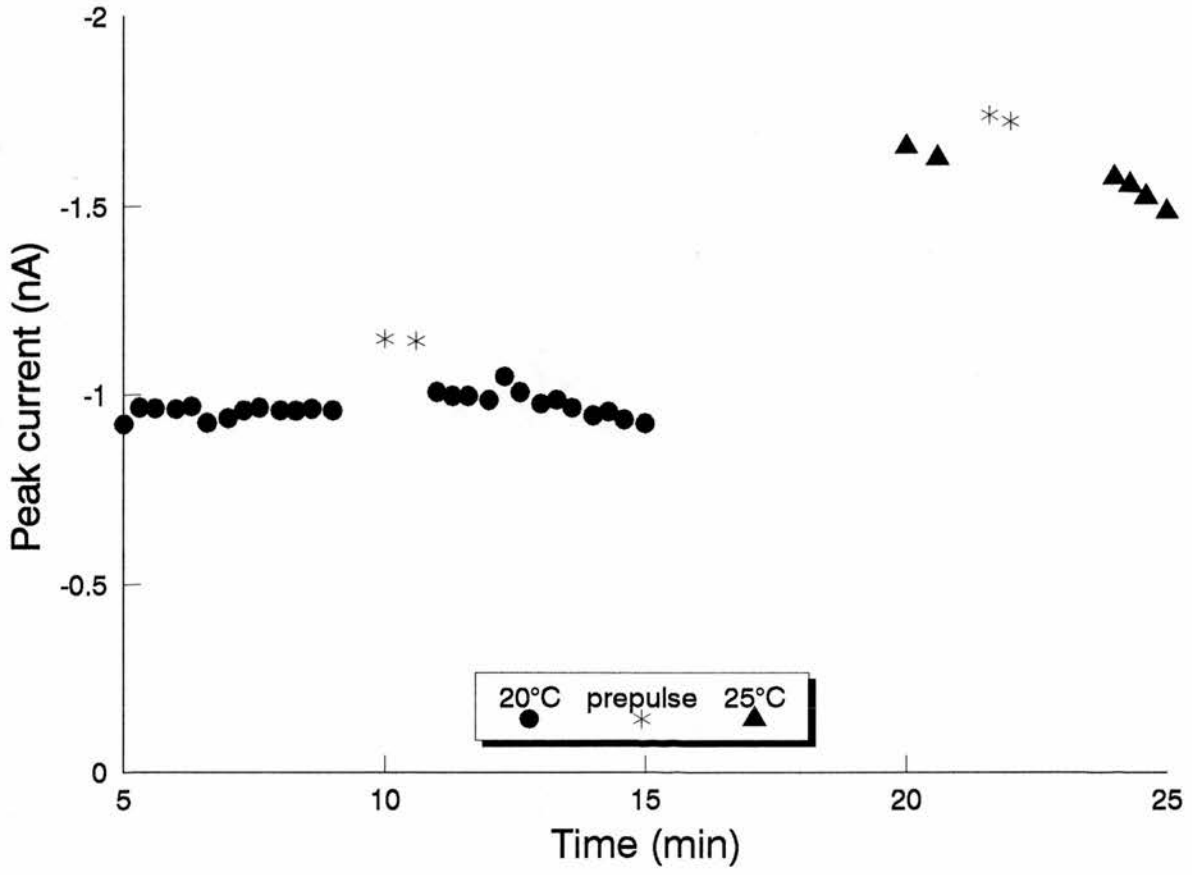

Figure 3.15: "Run-down" of the peak calcium current in the presence of GTP-γ-S. Peak Ca current elicited in the presence of GTP-γ-S (200 μM) with standard voltage steps at 20° C (●) and 25° C (▲) is shown in relation with time. At 20° C the maximal peak current was observed, on average, in 11 min (n= 16) after obtaining a whole-cell mode, rather later than in control cells (mean= 7.6 min). "Run-down" was smaller than in control conditions, and 30 min later the peak current amplitude decreased by 5.6%. With an increase in temperature by 5° C the current was significantly potentiated to 159% of control and "run-down" was faster. Application of a prepulse (✱) at both 20° and 25° C additionally potentiated the peak current. Currents are leak and capacity subtracted. GTP-γ-S was included in the pipette solution and standard external solution was used. Cell 1703931.

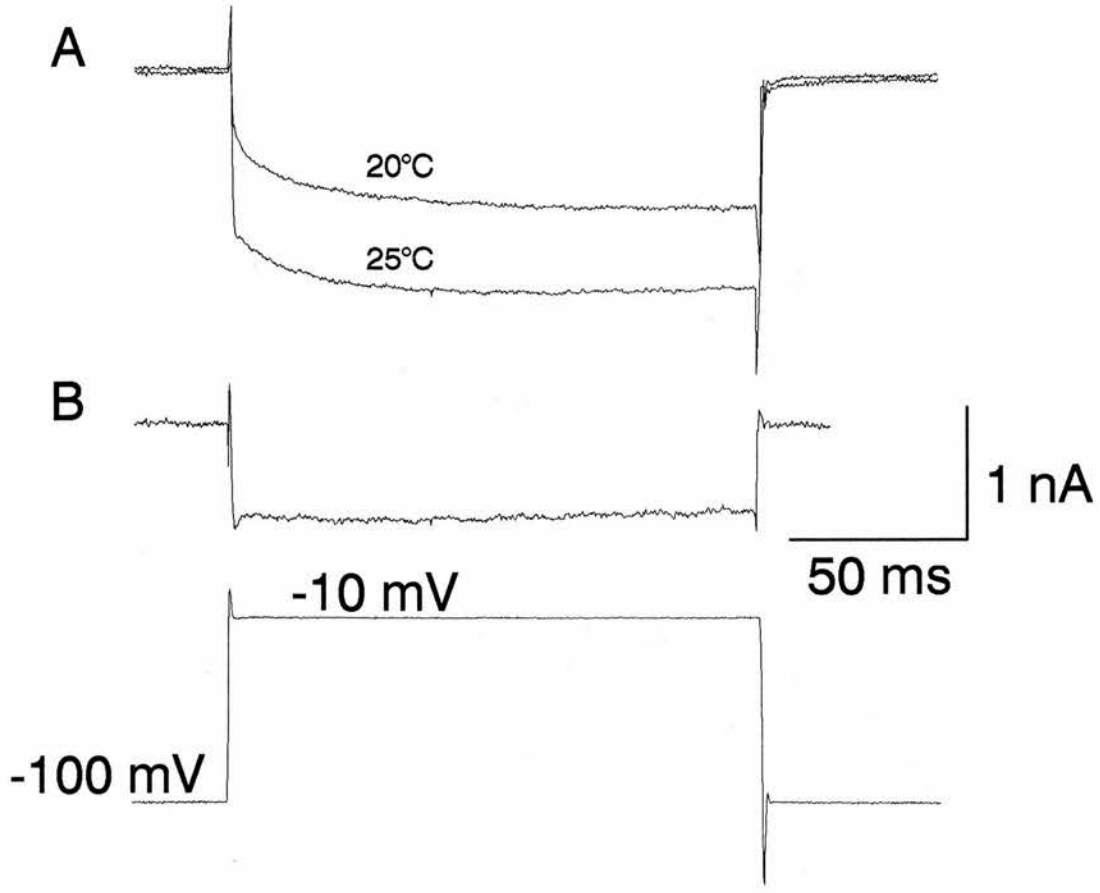

Figure 3.16: Temperature-dependency of the peak calcium current in DR neurones dialysed with GTP-γ-S. Ca current evoked at 20° C in the cells dialysed with 200 μM GTP-γ-S peaked, on average, at 1.02 nA (n= 16), significantly lower than in control experiments (1.82 nA, $p< 0.001$), and mimicking the peak current amplitude observed in the presence of 8-OH DPAT (0.98 nA). With a temperature step to 25° C, the current was significantly enhanced, on average, to 1.68 nA (n= 2) and the effect of temperature was fully reversible. In (A) superimposed peak Ca current traces are shown at 20° and 25° C, respectively, and in (B) the current component is shown that was potentiated with an increase in temperature. Peak currents were evoked using standard voltage protocol (lower panel), and GTP-γ-S was included in the recording pipette. Currents are leak and capacity subtracted. Standard external solution was used. Cell 1703932.

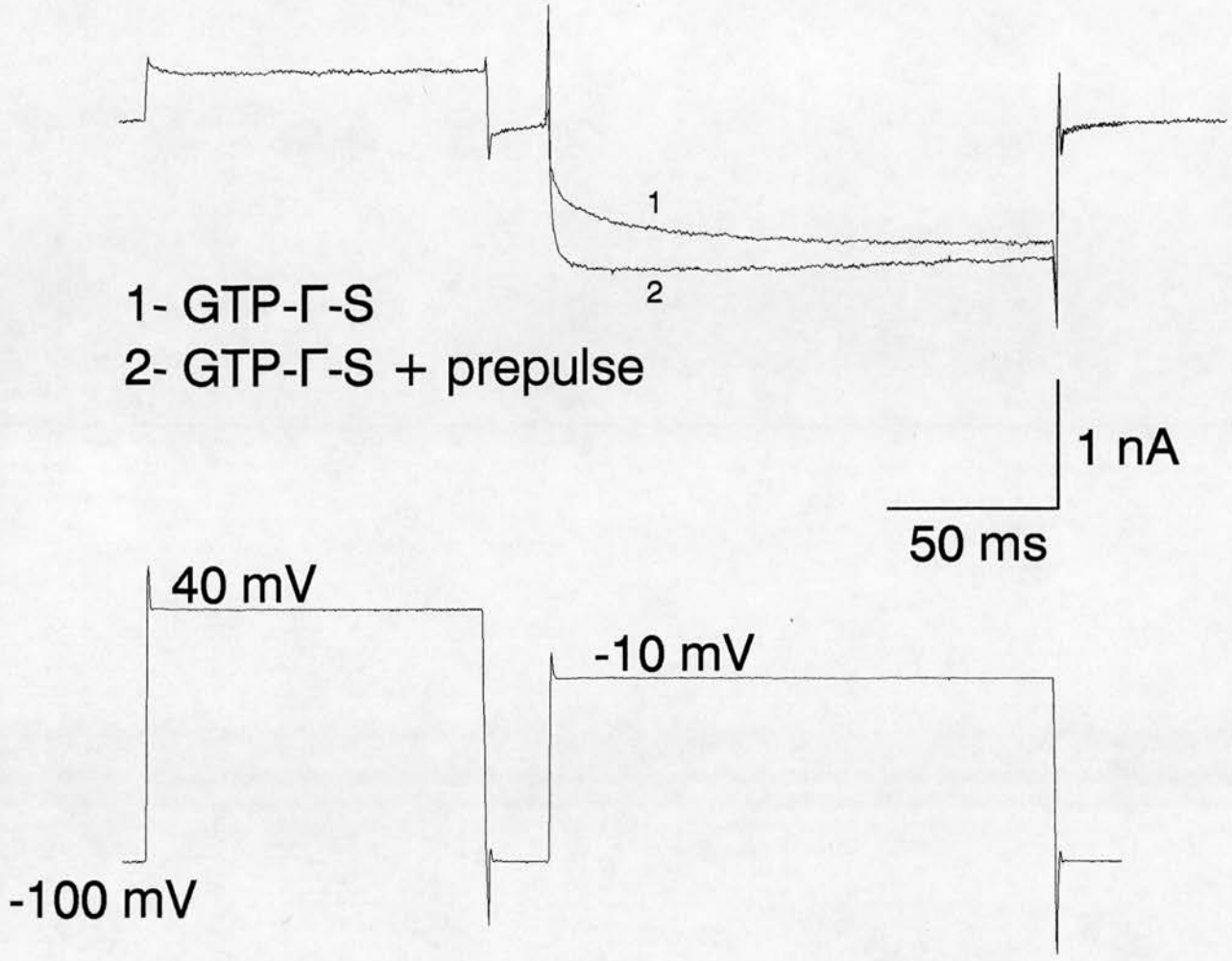

Figure 3.17: Prepulse substantially relieved the inhibition of the peak calcium current in DR neurones dialysed with GTP-γ-S at 20°C. At 20°C Ca current peaked, on average, at 1.02 nA in the presence of GTP-γ-S (200 μM, 1), and application of a prepulse (2) partially relieved the inhibition of the peak current. With the prepulse, the current was potentiated, on average, to 1.11 nA (n= 16). Voltage pulses are shown in the lower panel. GTP-γ-S was included in the internal solution and standard internal and external solutions were used. Currents are leak and capacity subtracted. Cell 0804931.

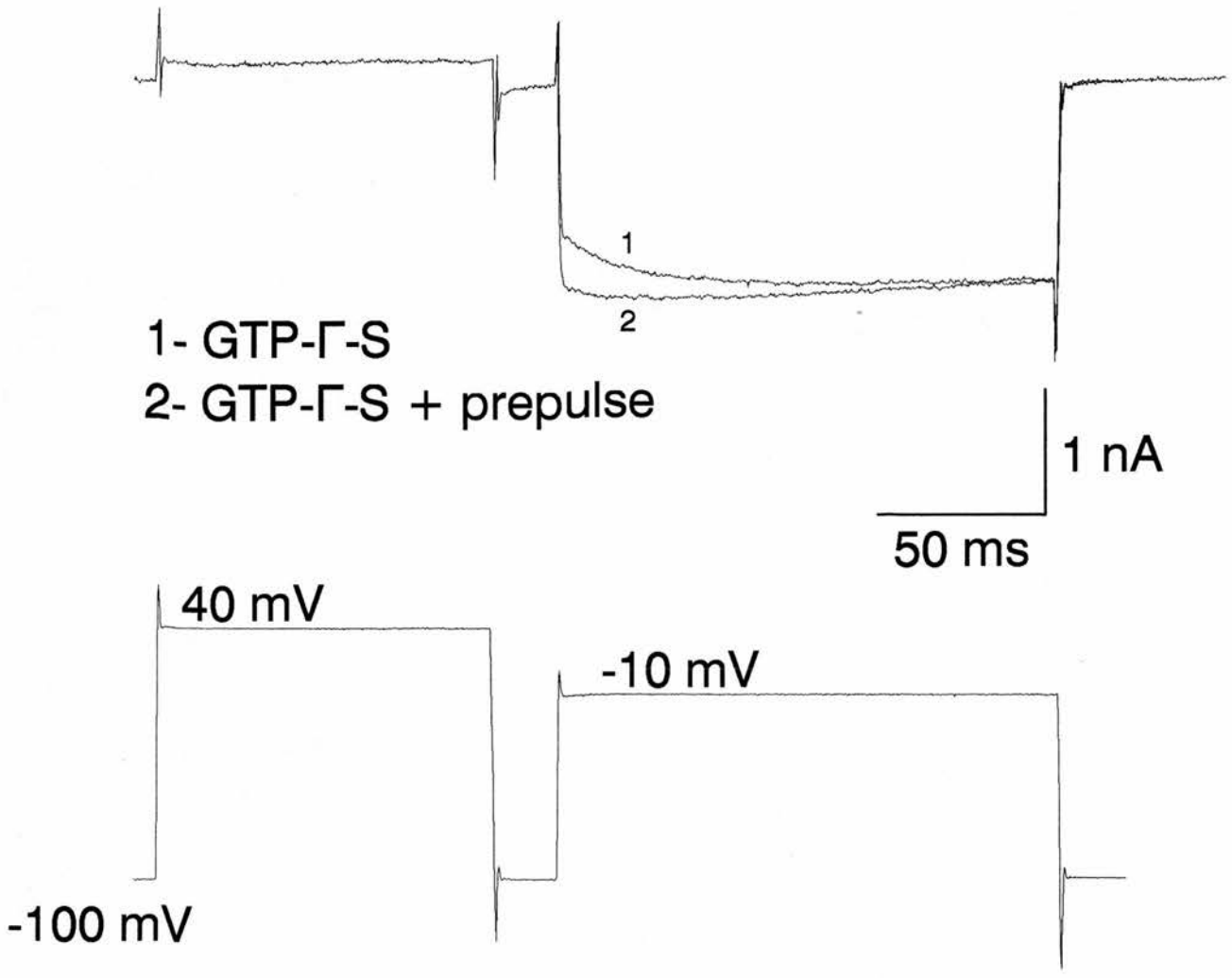

Figure 3.18: Potentiation of the peak calcium current and effect of a prepulse in DR neurones dialysed with GTP-γ-S at 25°C. At 25°C Ca current peaked, on average, at 1.68 nA and in the presence of a prepulse was enhanced to 1.73 nA, n= 3. GTP-γ-S (200 μM) was added to the internal solution. Standard internal and external solutions were used. Currents are leak and capacity subtracted. Voltage pulses are shown in the lower panel. Cell 0804932.

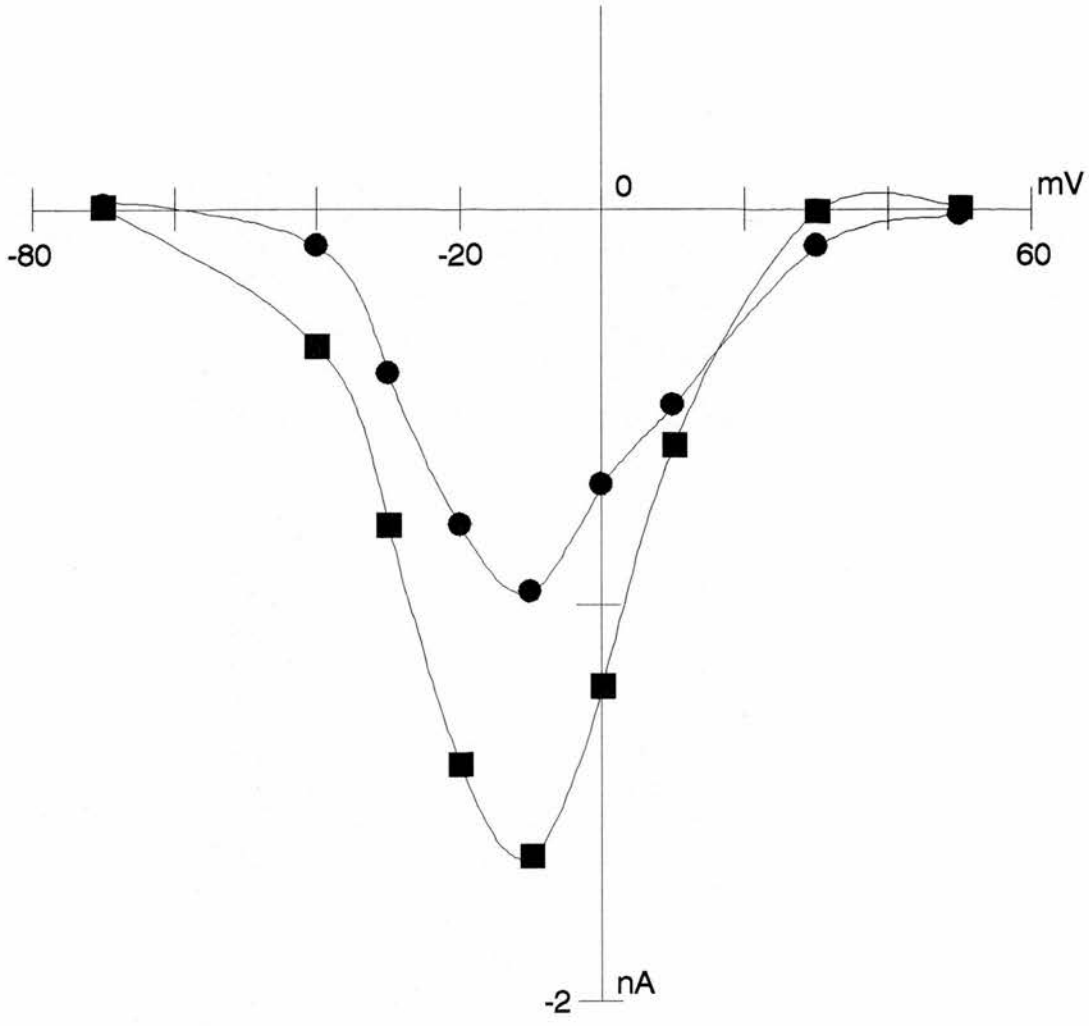

Figure 3.19: Voltage-dependency of calcium currents evoked in the presence of GTP-γ-S. Ca currents evoked from a holding potential of -100 mV are plotted against various test potentials. The currents at both 20°C (●) and 25°C (■) were voltage-dependent, activated at -40 mV and peaked at around -10 mV. Reversal potential was at 50 mV, as in control cells. The currents elicited at both temperatures were best differentiated at the peak potential of -10 mV, whereas at more positive potentials they were of similar size. The curve was fitted using a cubic spline routine. Currents are leak and capacity subtracted. Standard internal and external solutions were used. Cell 1703933.

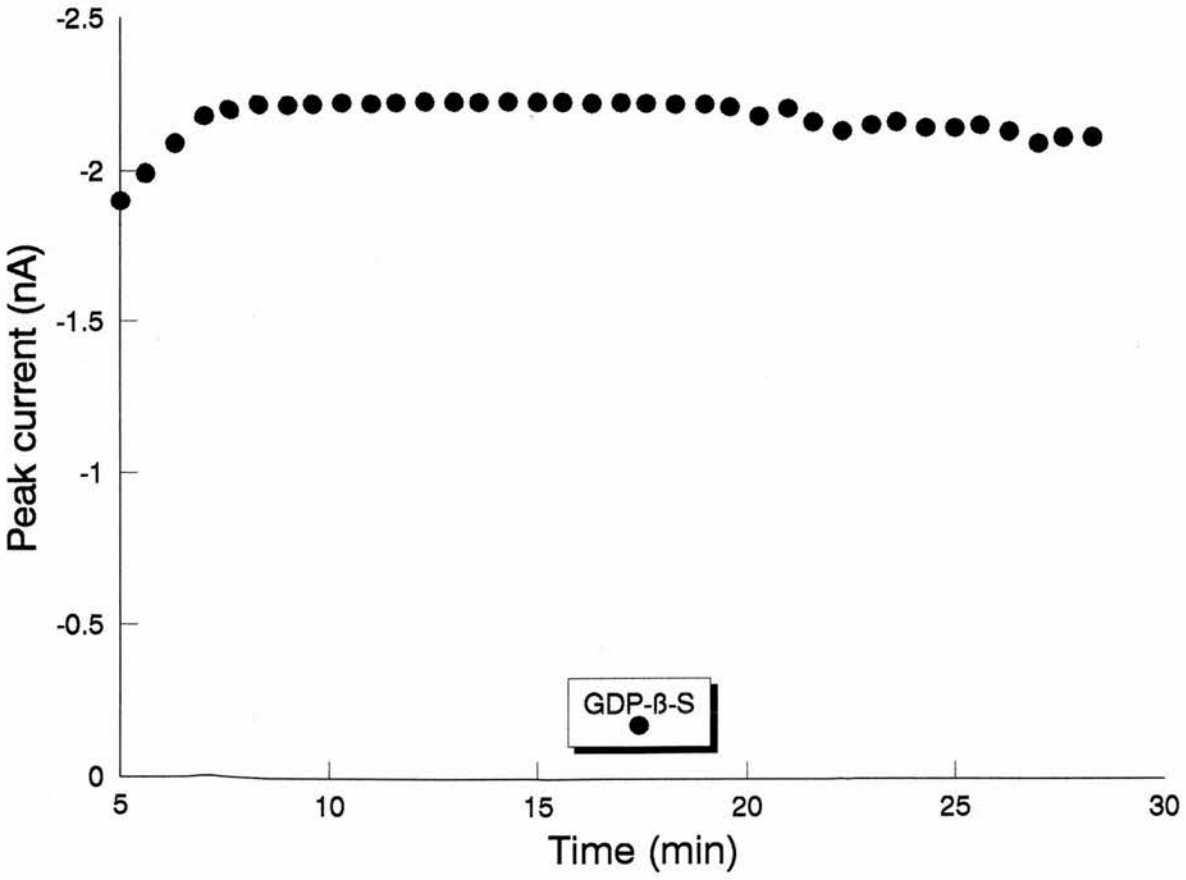

Figure 3.20: "Run-down" of the peak calcium current in DR neurones dialysed with GDP-β-S. GDP-β-S (2 mM, ●), a compound that inhibits G-protein activation, was included in the recording pipette. Ca current peaked, on average, at 1.89 nA, n= 4, that is similar to the peak current evoked in control DR neurones (1.82 nA). "Run-down" was not significantly different than in control situation and 20 min after obtaining the maximal amplitude, the current decreased, on average, by 14%. Currents are leak and capacity subtracted. Standard internal and external solutions were used. Cell 1606931.

Chapter 4 Effect of 5-HT_{1A} receptor activation on calcium current kinetics

Effect of 5-HT_{1A} receptor activation on calcium current kinetics

In DR neurones, it was quite apparent that an application of the 5-HT_{1A} agonist or G-protein stimulation, resulted in a change in Ca current kinetics. Therefore, it was challenging to examine the nature of the Ca currents modulation in various situations. It has been shown that in many other neuronal cell types, a modulation of Ca current by neurotransmitters also affects the current kinetics (Brown et al. 1981; Fenwick et al. 1982; Byerly et al. 1984; Kay & Wong, 1987; Kay, 1991; Allen et al. 1993).

Calcium currents activation

To obtain a clearer picture of the effects of 5-HT_{1A} receptor stimulation on DR neurones, it was necessary to examine the kinetics of activation. This has been performed by applying available mathematical functions and determining the best fit to the Ca current activation, elicited with depolarising pulses, using the standard voltage protocol. Activation of the current was analysed from the time of the voltage step to the time the current peaked, following leak and capacity subtraction. Peak current amplitudes were obtained with voltage steps from V_H -100 mV to -10 mV, for 150 ms. Single and double exponential functions and Hodgkin and Huxley models, with the analysis of m^1 - m^4 fits (see *Methods*) were used.

In Figure 4.1 (A) the activation of Ca current in a representative control DR neurone, at 20° C is shown, together with the best fit. In 75% of the control cells (n= 34), the best fit was a single exponential, with an activation time constant, τ_a, of 2.35±0.22 ms. When the same cell was held at 25° C activation was much faster than at 20° C. That result is, to a certain degree, discrepant with the results from other

groups. For instance, Fenwick et al. (1982) showed that in cultured chromaffin cells, at 20° C, the optimal fit was a sum of three components. Allen et al. (1993) suggested that the activation of the HVA Ca current in rat magnocellular cholinergic basal forebrain neurones could be described by a double exponential fit with the time constants $\tau_{a,f}$ and $\tau_{a,s}$ of 16 and 301 ms, respectively. However, the current data are in agreement with the observations of Brown et al. (1981) in *Helix aspersa* neurones, who fitted the Ca current activation using a monoexponential equation. It is possible that the problem might be in the fact that the sensitivity of the instruments that other groups used (Kay & Wong, 1987) was able to resolve the activation time at a resolution of ~0.1 ms, whereas the settling time of the voltage-clamp used in the present study, was 0.5-1 ms. Therefore, it was difficult to analyse brief, fast components of activation. In addition, Allen et al. (1993) used Ca^{2+} as the charge carrier, whereas Ba^{2+} ions were used in the present experiments.

In a few cells, there was only a small, almost negligible, difference between a single and double exponential fits. A good example of that is shown in Figure 4.1 (B). Both fits illustrated here have almost equal ability to fit the activation curve. In this cell, the time constant for a single exponential fit was 2.34 ms, while $\tau_{a,f}$ and $\tau_{a,s}$ were 1.32 and 3.72 ms, respectively.

Hodgkin and Huxley model was also used to examine the activation kinetics. In control cells, the best fit was obtained with m^2, and in 40% of the neurones, m^1 fitted better. This is in accord with the reports from other laboratories (Brown et al. 1983; Byerly et al. 1984; Kay & Wong, 1987). At 20° C, the τ_as for m^1 to m^4 were 2.55, 2.48, 2.33 and 2.26 ms, respectively.

Effect of temperature and cadmium on activation kinetics

From a holding potential of -100 mV, a temperature dependency of the peak Ca current was studied in the same DR neurone. Temperature was tested at 15°, 20°, 25° and 30°C, see Figure 4.2. Time constant of activation was temperature dependent and the higher the temperature, the faster the activation. The same observation was reported by Byerly et al. (1984) in snail neurones and VanLunteren et al. (1993) in bullfrog sympathetic neurones. The best fit obtained at 20°C, was, as explained above, a single exponential, but at 15°C, a double exponential function was better.

Earlier it was shown that 0.1 mM of Cd^{2+} blocked most of the peak current. When the activation kinetics was analysed, it was found that Cd^{2+} also prolonged the activation time. Indeed, as illustrated in Figure 4.3, in the presence of Cd^{2+}, the current activation was best fitted by a double exponential. Following the removal of Cd^{2+} from the standard external solution, the activation kinetics was best fitted by a single exponential. Time constants for single exponential fits for control, Cd^{2+} and the current after the removal of Cd^{2+} were 2.33, 7.53 and 6.01 ms, respectively.

Effect of 5-HT_{1A} agonist on activation kinetics

There is an evidence to suggest that an application of an agonist suppresses Ca current and simultaneously slows the activation time (Elmslie, 1992; VanLunteren et al. 1993).

For that reason, it was important to examine the effect of 8-OH DPAT on the peak current kinetics in DR neurones. Figure 4.4 shows traces of the activated peak current

in control conditions, during the application of 8-OH DPAT and partial recovery. For control cells, τ_a was 2.35±0.22 ms and the best fit was a single exponential. However, perfusion of the cells with 8-OH DPAT (50 μM), produced a significant change in the activation kinetics, n= 22, and the time constant for a single exponential fit was 5.75±0.38 ms, i.e. a 2.5-fold increase compared to control cells. For a double exponential fit, which was the best fit in 72% of cells tested in the presence of 8-OH DPAT, the fast time constant, $\tau_{a,f}$, was 4.77 ms, and the slow, $\tau_{a,s}$, 6.08 ms. The m^1, rather than m^2 was the optimal relation for Hodgkin and Huxley model.

Following the administration of Cd^{2+} (0.1 mM) in the extracellular solution, to the cell still perfused with 8-OH DPAT (50 μM) a further increase in the activation time constant was seen, Figure 4.5. Comparing single exponential fits, the time constant in addition of Cd^{2+}, increased from 5.05 to 6.40 ms. Wash-out of Cd^{2+} partially restored the kinetics, n= 3.

Effect of prepulse on activation kinetics

In control cells, application of a prepulse had no effect on the peak current amplitude. Moreover, the prepulse had no effect on activation kinetics. In the presence of a prepulse τ_a was of 2.42±0.08 ms, compared to 2.35±0.22 ms in control cells. The best fit was a single exponential and m^2 was the best fit for the Hodgkin and Huxley model.

Ca current activation was almost entirely restored by a prepulse in cells bathed in 8-OH DPAT (50 μM), see Figure 4.6. The prepulse restored activation kinetics, that was again best fitted by a single exponential and m^2 was the best fit for the Hodgkin

and Huxley model. The time constant, τ_a, in the presence of 8-OH DPAT was 5.78±0.38 ms, and τ_a was of 2.60±0.15 ms with an addition of the prepulse. The result was very close to the activation kinetics and τ_a of 2.35±0.22 ms, prior to the 8-OH DPAT administration.

Effect of GTP-γ-S on activation kinetics

The effect of GTP-γ-S on the peak Ca channel current was almost identical to that produced in the presence of 8-OH DPAT. Therefore, it was of interest to examine the effect of GTP-γ-S on the current activation. Two effects were examined: firstly, to determine whether the activation was best fitted by a double exponential, as in the presence of 8-OH DPAT, at 20° and 25°C, and secondly, to see whether the prepulse restored the current activation. Figure 4.7 (A) shows that in the cells dialysed with 200 μM GTP-γ-S, n= 10, at 20°C, the best fit was a double exponential. In addition, m^1 was the best in 9 out of 10 cells. The prepulse had the same effect as seen in 8-OH DPAT cells; it completely restored the current activation, with τ_a of 2.64±0.09 ms, as in control cells. Single exponential fit was best in 78% cells (7/9).

Similar result was obtained at higher temperature, when DR neurones were held at 25°C (n= 3), in the presence of GTP-γ-S included in the standard intracellular solution, see Figure 4.7 (B). In the absence of a prepulse, the activation of the current was best fitted by a double exponential.

A prepulse restored the activation kinetics to a single exponential. It was some what unexpected that in the presence of GTP-γ-S, the peak current was best fitted by a double exponential at 25°C, because at higher temperatures the current activation is usually best fitted by a single exponential.

Calcium currents inactivation

The process of inactivation of Ca current has been examined by a number of workers (Brown et al. 1981; Chad & Eckert, 1986; Kay, 1991; Frace & Hartzell, 1993; VanLunteren et al. 1993). Kay (1991) described that the inactivation kinetics in acutely dissociated CA1 pyramidal neurones of guinea-pig hippocampus, was best fitted by a sum of two exponentials, with time constants of 200 ms and 2s. He suggested that inactivation results from a dual process of voltage- and Ca-dependent inactivation.

Inactivation of Ca channel current has also been studied in DR neurones. As discussed previously, inactivation was not seen in all cells and, when observed, it was very variable. A similar observation was made by Fenwick et al. (1983) in cultured chromaffin cells and Werz et al. (1993) in sympathetic neurones. In most DR neurones at 20° C, under control conditions, the current amplitude at the end of a 150 ms long test pulse was not significantly smaller than the peak current, and measured 1.75 nA compared to 1.82 nA, respectively. At 25° C, the inactivation kinetics was more apparent and the analysis was possible. The results shown here were obtained by eliciting the peak Ca current from holding potential of -100 mV to -10 mV, where the inactivation of the current was maximal. With the peak current elicited from V_H -60 mV, no inactivation was seen. The inactivation was analysed by fitting curves from the time the current peaked, to the end of the test pulse.

The current kinetics were strongly temperature dependent. Earlier, it was shown that with an increase in temperature the Ca current peaked at a higher amplitude and the activation time was shorter. Increasing the temperature from 20° C to 25° C, potentiated inactivation of the current in DR neurones. At 20° C, at the end of the test pulse, the current was 3.9% smaller than the peak current, whereas at 25° C that was 15%.

Figure 4.8 shows the effect of temperature on the peak current inactivation. A

single exponential was the best fit for inactivation at 20° C in all cells tested, n= 24. On the other hand, an increase in inactivation, produced at 25° C, was best fitted by both a single, n= 2, and double, n= 2, functions. This result might be in opposition to the report by Brown et al. (1981) and Kay (1991). In CA1 hippocampal neurones, Kay recorded that a double exponential was the best fit. He also suggested that inactivation was strongly Ca-dependent and used 10 mM BAPTA in the recording electrodes. In the present experiment 11 mM EGTA was used, which is weaker Ca chelator. Werz et al. (1993) also favoured the sum of two exponentials, but measured only the first 50 ms of inactivation of the inward current. In DR neurones, the marked change in inactivation rate was best demonstrated by a difference in the time constants. At 20° C, the peak current inactivated with a time constant of 203±31 ms, whereas at 25° C that was of 47±7.6 ms, an almost 5-fold speeding up of the inactivation.

Application of a prepulse had no effect on the inactivation time at 20° C. Single exponential was the best fit in all cells, n= 18. The time constant in the absence of the prepulse was 203 ms and 221 ms in its presence.

Only very few cells showed inactivation of the peak Ca current in the presence of 8-OH DPAT or GTP-γ-S. Indeed, as described earlier, in a number of cells dialysed with GTP-γ-S, Ca current did not reach a peak during a 150 ms long test pulse. As a result, the inactivation kinetics was not measured in such small number of cells.

Calcium currents deactivation

Tail currents can be used to analyse deactivation of Ca current, when the

membrane potential is returned to its holding value. Single and double exponentials were used to fit the current deactivation. Tail current was determined from the time the membrane holding potential settled at -100 mV, until the current decayed fully. In majority of the neurones tested, the tail currents were difficult to separate from leakage currents. An excessive variability of tail currents was present from cell to cell. For that reason, the results obtained in the presence of a prepulse, and at 25° C, are not illustrated in this study. The other reason is, as it was described for the activation kinetics, that we experienced a relatively slow settling time of the voltage-clamp. In most cases, the tail currents were of very rapid nature.

Neurones were tested at 20° C exactly. In 80% of cells (12/15), the tail current was best fitted by a single exponential. In another three cells, the best fit was a double exponential. Some laboratories have reported the existence of an additional component, or have used a double exponential fit (Fenwick et al. 1982; Byerly et al. 1984). In DR neurones, the time constant for the tail current was of 2.77±0.24 ms. This is in line with the observation in sympathetic ganglion neurones by Sala (1991). As in the analysis of the fast rate of activation in DR neurones, it was possible that the fast component of the tail current was not detected or separated from the capacitance artifact.

The effect of 8-OH DPAT and GTP-γ-S was also studied. An impressive observation was, that both compounds led to a prolongation of the tail current. The tail currents were best fitted by a double exponential. Time constants, τ_t, for single exponentials for 8-OH DPAT and GTP-γ-S was 3.65 and 3.79 ms, respectively. The result was significantly different than that in the control experiments, with time constant of 2.77 ms ($p < 0.01$).

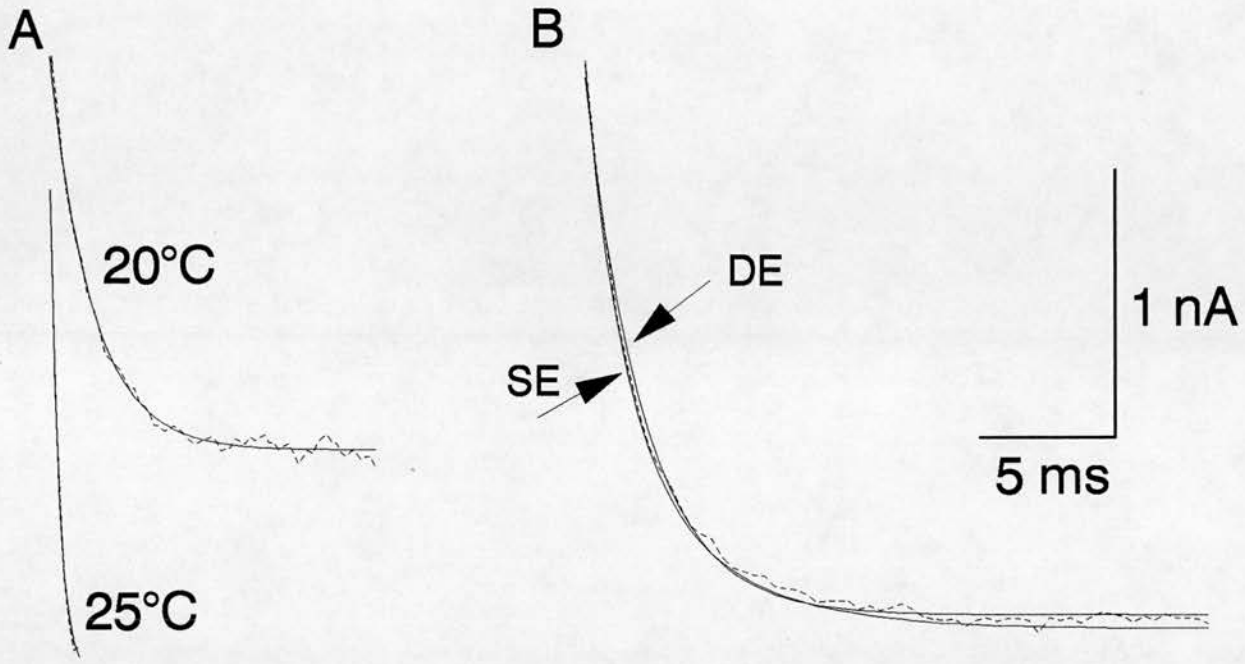

Figure 4.1: Activation kinetics of the peak calcium current at 20° and 25°C. Peak Ca current was evoked in a typical DR neurone and shown as a broken line (···), together with the fitted curve (—). At 20°C, the activation time constant, τ_a, was, on average, of 2.35 ms (n= 34), whereas at 25°C, the activation was faster, n= 4. The best fit for the current activation at both 20° and 25°C was a single exponential (A). In minority of DR neurones (5.1%), both a single and double exponential showed an equal ability to fit the activation curve, as shown in (B). Standard voltage steps were used and currents are leak and capacity subtracted. Standard internal and external solutions were used. SE= single exponential; DE= double exponential. Cells 2101932 (A) and 2601931 (B).

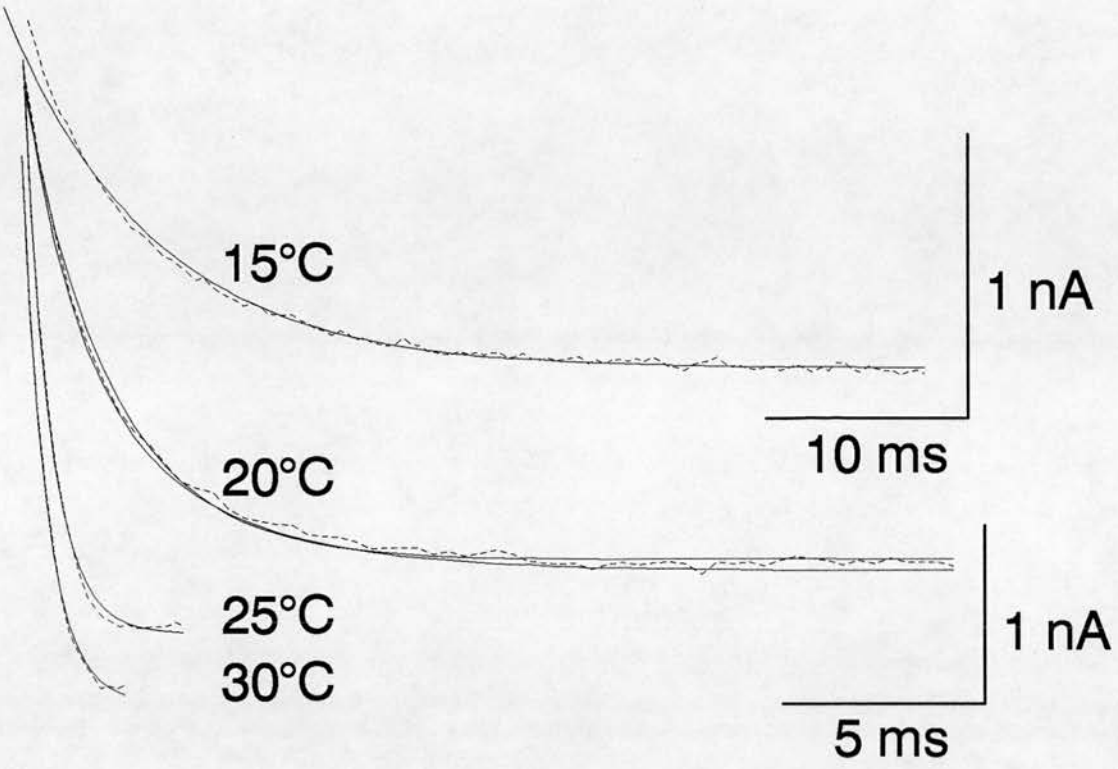

Figure 4.2: Effect of temperature on the activation kinetics in a representative DR neurone. Activation kinetics of the peak Ca current was temperature-dependent. At 15°C, the best fit was a double exponential, whereas at 20°, 25° and 30°C, the best fit was obtained with single exponentials. Currents, evoked with the standard pulses, were leak and capacity subtracted and standard internal and external solutions were used. Two calibration bars are shown, the upper one for the current evoked at 15°C, and the lower one for the other currents shown. Cell 2201931.

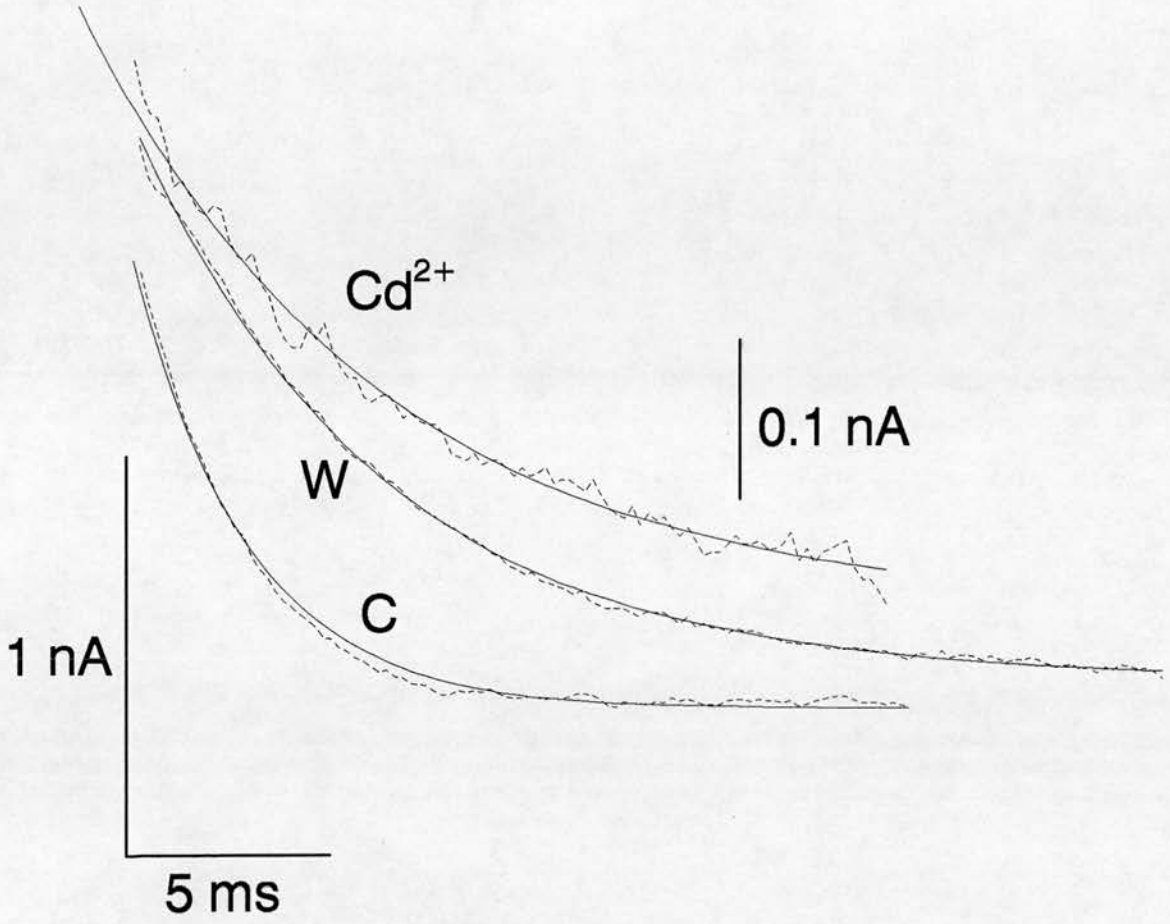

Figure 4.3: Effect of cadmium on the peak calcium current. Cd^{2+} (0.1 mM) was applied externally, close to the DR neurone and prolonged the activation of the peak Ca current. In the presence of Cd^{2+}, the best fit was a double exponential, and following wash-out of the drug the activation kinetics was best fitted by a single exponential, as in control situation. Time constants (τ_a) for single exponential fits for control current (C), current evoked in the presence of Cd^{2+} and following wash-out (W) of Cd^{2+} were, on average, 2.33, 7.53 and 6.01 ms, respectively. Two calibration bars for the current are shown; the upper one for the current evoked in the presence of Cd^{2+}, and the lower one for the other currents shown. Cell 2001931.

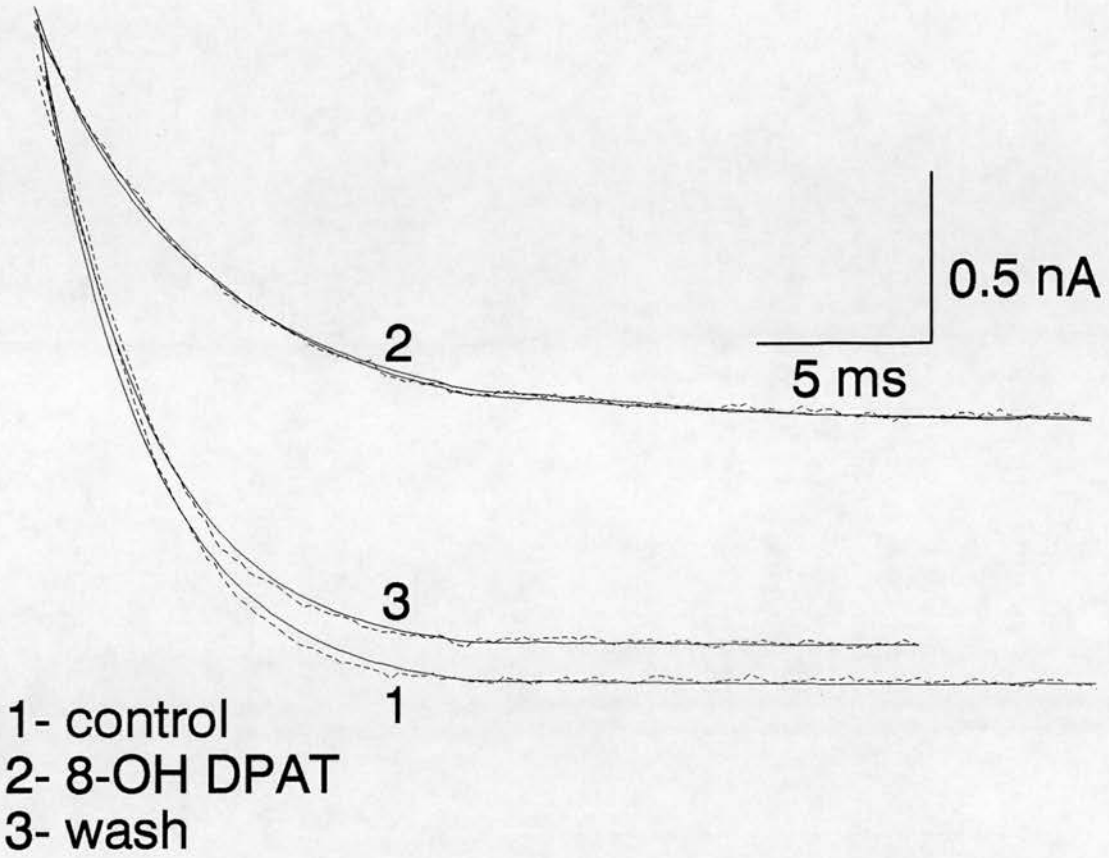

Figure 4.4: 8-OH DPAT substantially prolonged the activation time of the peak calcium current. Peak Ca current in control situation (1) activated with a time constant of 2.35 ms and a single exponential was the best fit. Extracellularly applied 8-OH DPAT (50 μM, 2) dramatically increased the activation time by 2.5 fold. In 72% of cells the best fit for the activation of the peak Ca current in the presence of 8-OH DPAT was a double exponential with fast, $\tau_{a,f}$, and slow, $\tau_{a,s}$, time constants of 4.77 and 6.08 ms, respectively, n= 22. Following wash-out (W) of the drug, the best fit was again a single exponential. Cell 0903932.

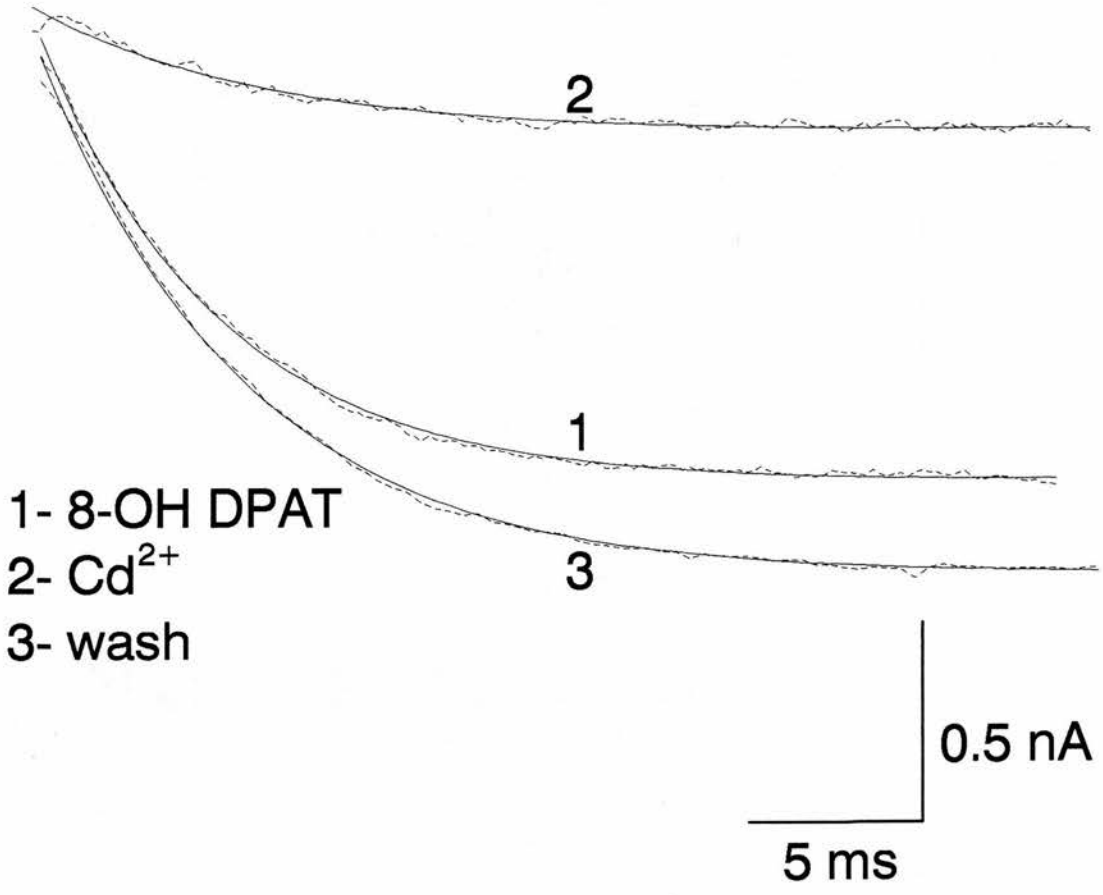

Figure 4.5: Effect of cadmium on the activation kinetics in the presence of 8-OH DPAT. Cd^{2+} (0.1 mM) was applied externally into the vicinity of the DR neurone that was continuously perfused with 8-OH DPAT (50 μM). A double exponential was the best fit in the presence of both drugs. Time constants of activation during perfusion with 8-OH DPAT (1) and with both 8-OH DPAT and Cd^{2+} (2) had $\tau_{a,f}$ of 4.77 and 5.05 ms, and $\tau_{a,s}$ of 6.39 and 5.06 ms, respectively. The effect of Cd^{2+} was fully reversible following wash-out (3). Cell 1203931.

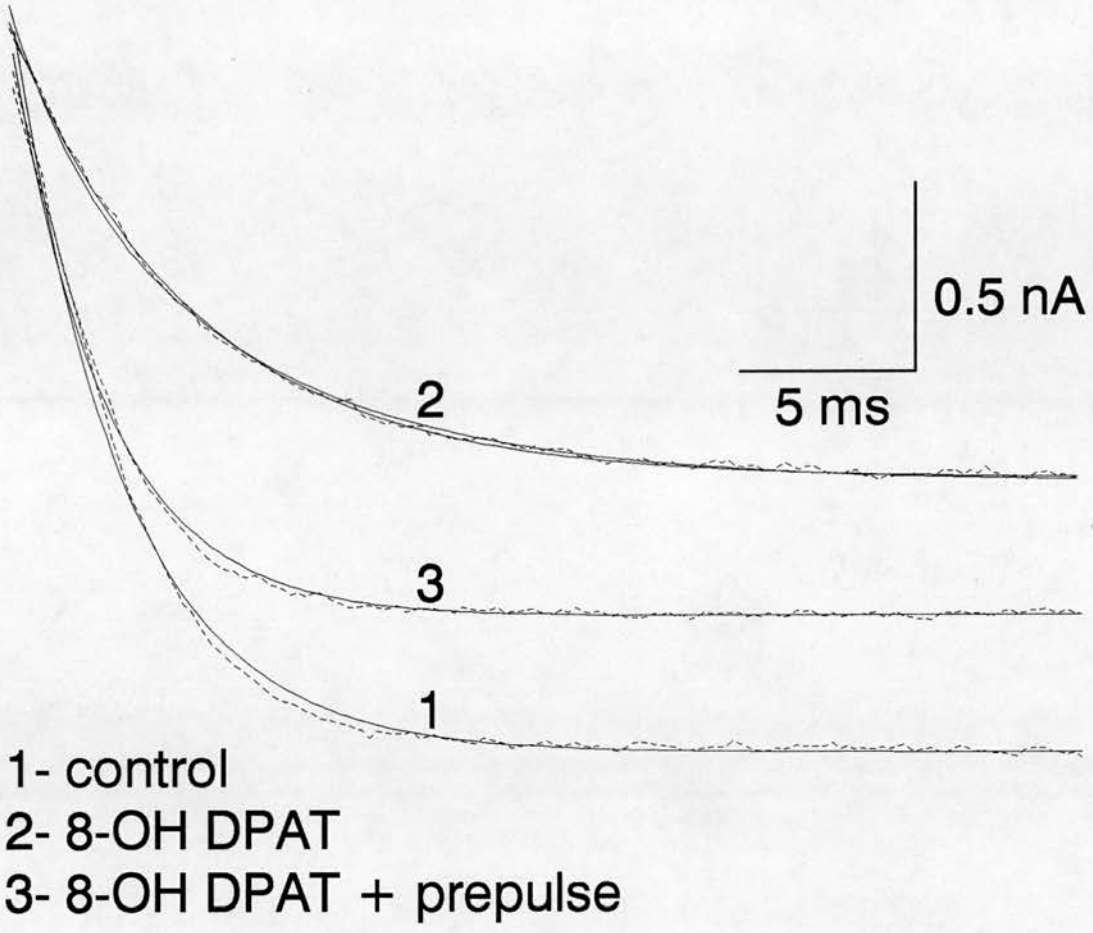

Figure 4.6: Prepulse completely restored the activation kinetics in the presence of 8-OH DPAT. Peak Ca current in control situation (1) was evoked using a standard voltage protocol and the best fit was a single exponential. Application of 8-OH DPAT (2) into the external solution slowed the activation of the peak current and the best fit was a double exponential. In addition of a prepulse (3), the activation kinetics was restored and the best fit was a single exponential. Time constants of activation in control cells, in the presence of 8-OH DPAT and in addition of the prepulse were of 2.35, 5.78 and 2.60 ms, respectively. Cell 0903932.

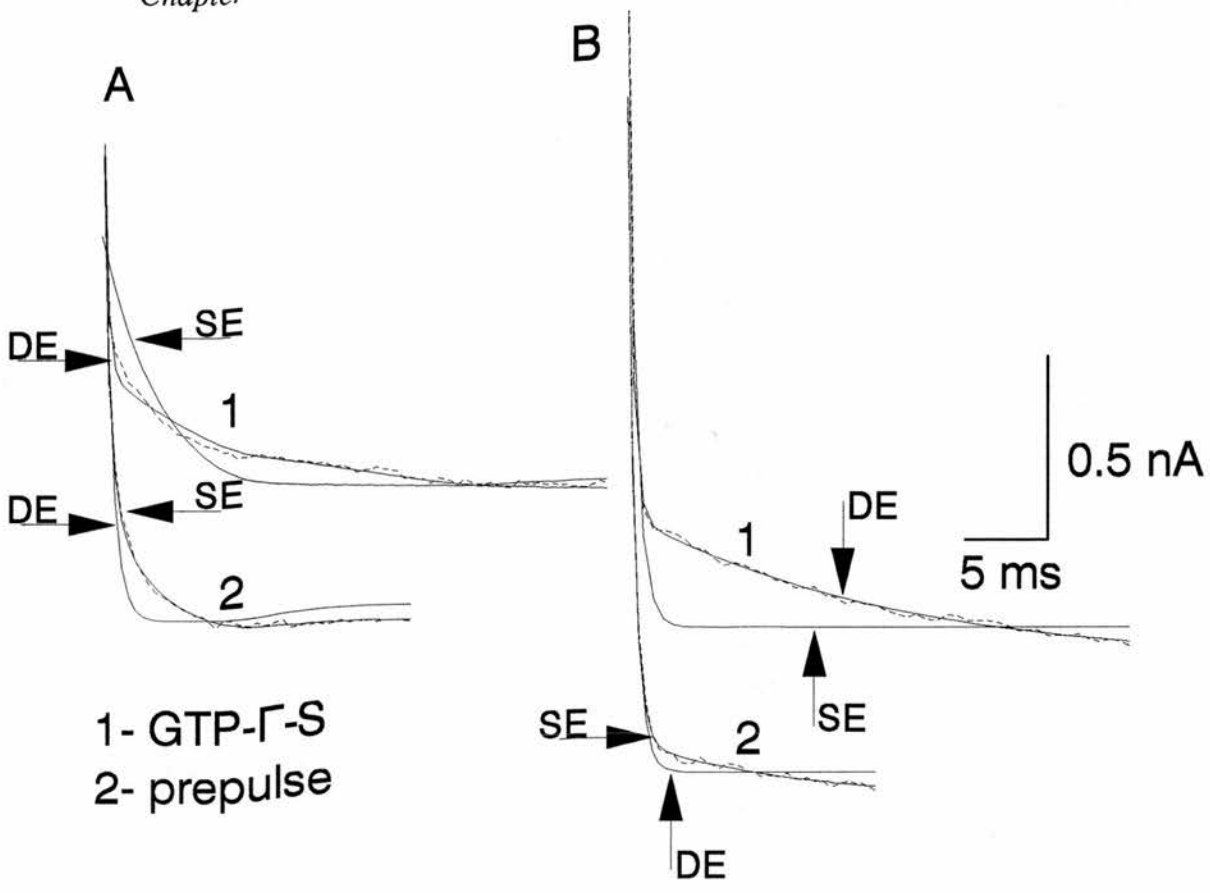

Figure 4.7: Effect of a prepulse on the activation kinetics of the peak calcium current in the cells dialysed with GTP-γ-S, at 20° and 25°C. In DR neurones intracellular application of 200 μM GTP-γ-S had similar effect on the activation kinetics as 8-OH DPAT. The best fit was a double exponential at 20° and 25°C (1), respectively. In addition of a prepulse (2), the activation kinetics was restored in 7/9 neurones, that is the best fit was a single exponential with the time constant, on average, of 2.64 ms (A), as in control cells. Similar observation was made at 25°C (B). Both fits, a single (SE) and double (DE) exponential are superimposed over the current trace. Cells 0804931 (A) and 0804932 (B).

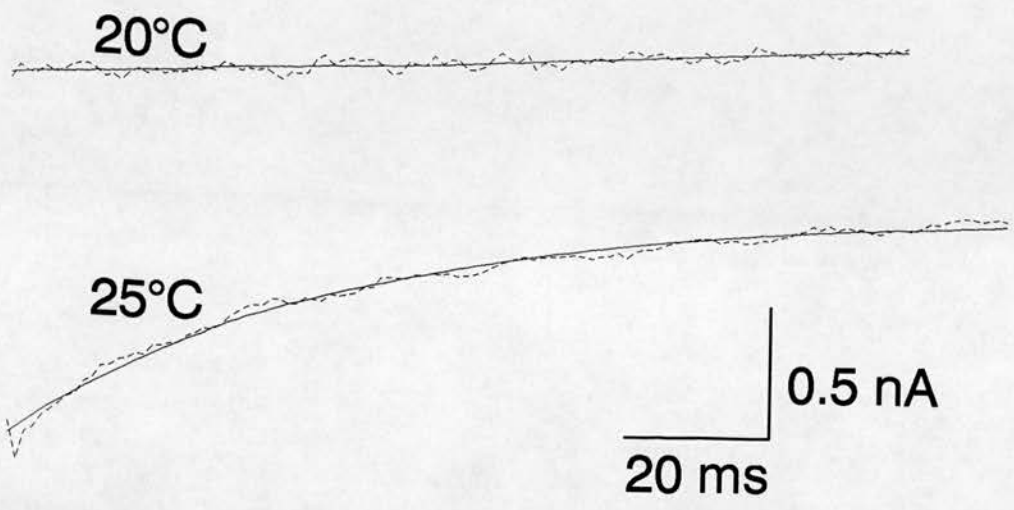

Figure 4.8: Inactivation of the peak calcium current at 20° and 25°C. At 20°C inactivation was best fitted by a single exponential in all neurones studied (n= 24), whereas at 25°C, both a single (2/4) and double (2/4) exponentials were the best fit in the same number of cells. The peak Ca current inactivated with τ_i of, on average, 203 ms at 20°C, and an increase in temperature for 5°C produced a 5-fold speeding up of the inactivation. Cell 2201934.

Chapter 5 Effect of protein phosphatase inhibition on calcium currents

Effect of okadaic acid on calcium currents

The involvement of phosphorylation in the modulation of Ca currents was studied using the okadaic acid group of compounds.

These drugs are potent natural blockers of two serine/threonine protein phosphatases: protein phosphatase 1 (PP1) and phosphatase 2A (PP2A), (Ingebritsen & Cohen, 1983; Bialojan & Takai, 1988; Haystead et al. 1989).

Many groups have shown that phosphorylation is a necessary step in ion channel functioning (Armstrong & Eckert, 1987; Hescheler et al. 1988; Elmslie et al. 1993; Frace & Hartzell, 1993). When the effect of okadaic acid (OA) was examined on the HVA Ca current in DR neurones, the compound increased the peak Ca current and slowed "run-down" in 80% of DR neurones tested (36 out of 45 cells). Figure 5.1 compares the time course of the increase in the peak Ca current amplitude with time in a representative DR neurone dialysed with 1 μM OA to a control experiment, i.e. in the absence of OA. In all experiments the peak Ca current amplitude in the presence of protein phosphatase inhibitors was elicited with voltage pulses from V_H -100 mV to -10 mV for 150 ms, at a frequency of 0.05 Hz. Recording started 5 min after obtaining a whole-cell mode. In the presence of OA a maximal peak amplitude occurred, on average, in 10-18 min (mean 13.6 min, n= 45), rather later than in control experiments (mean= 7.6 min), after establishing the whole-cell configuration. Once the current stabilised, the average peak amplitude was 2.53±0.06 nA, 39% higher than in control cells. This is in an agreement with the reports from Hescheler et al. (1988) and Frace and Hartzell (1993). In their hands, OA (5-100 μM) was applied extracellularly and potentiated the peak HVA Ca current in guinea-pig and frog cardiomyocytes. Mironov and Lux (1991) also observed a potentiation of the HVA Ca current, when isolated rat hippocampal neurones were bathed in OA. Werz et al. (1993) reported that OA enhanced the peak Ca current in isolated sympathetic ganglion neurones.

Another, very striking, observation shown in Figure 5.1, is that OA, included in a recording pipette, slowed "run-down". The rate of "run-down" in OA dialysed cells was, on average, 8±0.9%, 30 min after obtaining the maximal peak amplitude. That was significantly different than in control cells (17%), $p< 0.01$. It appears that OA, by an inhibition of dephosphorylation stabilised Ca currents. This phenomenon has also been observed in other neuronal cells (Belles et al. 1988; Hescheler et al. 1988; Mironov & Lux, 1991; Reinhart et al. 1991; Artalejo et al. 1992b; Clark, 1993; Elmslie et al. 1993; Werz et al. 1993).

It was described earlier that in control experiments prepulses had no significant effect on the peak Ca current in DR neurones. However, the effect of a prepulse was more prominent in the presence of OA. A prepulse application potentiated the current, which on average peaked at 2.61±0.08 nA, n= 21, see Figure 5.2. Moreover, additional prepulses were still able to enhance the peak current, albeit to a lesser degree.

In another set of experiments (n= 3), OA (1 μM) was included only at the back part of the recording pipette by "back filling", see *Methods*. This allows OA to reach the cell and dialyse it more slowly by diffusion, as shown in Figure 5.3. A prolonged increase in the size of the peak current amplitude was observed, when OA was included in the back of the recording pipette, and the same rate of "run-down" as in control OA experiments was recorded. The maximal peak amplitude of the HVA Ca current was obtained some what later than in a control OA cell, see Figure 5.1. In these neurones, the maximal peak current was observed in 11-22 min (average 16 min), whereas in control OA cells, the median time was 13.6 min. Prepulse applied once the peak current had stabilised, potentiated the current to the same extent as in control OA neurones.

The current-voltage relationship in a typical DR cell dialysed with OA is illustrated

in Figure 5.4. As in control experiments, the currents were evoked from a holding potential of -100 mV and the threshold for the current activation was around -40 mV. Unexpectedly, the peak current amplitude was shifted to the left. Thus, there was a very steep increase in the size of the currents positive to -30 mV, and they peaked at around -20 mV. The same pattern was observed in all cells tested, n= 9. In the Figure, both transient and slowly decaying components of the Ca current are plotted and no significant inactivation of the current was observed at 20° C. OA treated cells reversed the potential at around 50 mV, as in control cells. In cardiac myocytes perfused with OA, Frace and Hartzell (1993) observed no shift in the peak Ca current nor in reversal potential.

It appears that phosphorylation is strongly temperature-dependent. Current traces obtained in the same neurone in the presence of OA, at 20° and 25° C, and evoked by standard voltage pulses are shown in Figure 5.5. In all five experiments an increase in temperature from 20° to 25° C enhanced the peak Ca current amplitude. At 20° C the current peaked at 2.53±0.06 nA, and a negligible inactivation of the current was observed. The temperature was then stepped to 25° C, and the current peaked, on average, at 3.23±0.11 nA, that was significantly higher than at 20° C, $p< 0.01$. Inactivation was much stronger at the higher temperature (A). Inset (B) shows a subtraction of the two currents, obtained at 20° and 25° C, respectively.

Effect of 8-OH DPAT in the presence of okadaic acid

Effect of the 5-HT_{1A} agonist 8-OH DPAT was studied in DR neurones dialysed with OA. Figure 5.6 shows a time course of the peak Ca current amplitude evoked in the presence of 1 μM OA. Once the current had stabilised, 8-OH DPAT (50 μM)

was applied externally to the cell during a period of time and inhibited the peak Ca current, on average, by 31±2.6%, n= 16. The second application, 30 min later, produced the same effect, albeit the inhibition was less. These experiments show that OA was unable to prevent the inhibition of the peak Ca current caused by the 5-HT_{1A} receptor activation. Furthermore, this would suggest that phosphorylation is not involved in the modulatory effect of 8-OH DPAT on the HVA Ca currents and does not share the same pathway of modulation with 8-OH DPAT. This observation is supported by the reports from other laboratories. Elmslie et al. (1993) showed that norepinephrine partially inhibited the peak Ca current and the effect was not prevented by an inclusion of OA in the recording pipette in bullfrog sympathetic neurones. Hescheler et al. (1988) made similar observation in cardiomyocytes during β-adrenergic stimulation.

The most dramatic effect observed in the experiments with 8-OH DPAT was that of a prepulse. A prepulse applied in the presence of OA and 8-OH DPAT reduced the inhibition of the peak Ca current caused by 8-OH DPAT by 45% and the current peaked, on average, at 1.82±0.05 nA. However, that was not the sole effect of the prepulse. Surprisingly, as in the DR neurones tested in the absence of OA, the prepulse fully restored the activation kinetics, see Figure 5.7.

It was necessary to confirm that the effects described, recorded in the presence of OA, resulted from the inhibition of protein phosphatase 1 and/or 2A. For that purpose, other PP1 and PP2A blockers were tested using the same protocol as in the experiments with OA. The results are presented in the following sections.

Effect of 1-norokadaone on calcium currents

It was reported that 1-norokadaone (1-NO) has similar chemical structure to OA, but does not inhibit PP1 and PP2A and can be used as an inactive analogue of OA (Nishiwaki et al. 1990). 1-NO (1 μM) was included into the recording pipette and its effect is shown in Figure 5.8. The peak Ca current was not enhanced in the presence of the compound as in OA cells, and at 20° C the peak current was maximal in 7-12 min, with a median of 10.3 min (n= 4), rather earlier compared to 13.6 min in the presence of OA.

Furthermore, "run-down" was not prevented and the peak Ca current decreased by 16%, 30 min later. A prepulse application had no effect on the peak current. With an increase in temperature to 25° C, the current amplitude was reversibly potentiated and peaked at 2.73 and 2.72 nA, n= 2. The prepulse produced only weak and reversible enhancement of the peak current, to 102% of control, at 25° C. Inset in the Figure shows the peak Ca current traces obtained in another cell perfused with 1-NO at 20° C at the beginning (1) and at the end (2) of the recording. "Run-down" was not prevented by the presence of 1-NO, as described above for okadaic acid.

Therefore, it appears that 1-norokadaone, an inactive analogue of OA, produced no significant change in the peak Ca current amplitude or the current kinetics, when compared to control DR neurones.

Effect of microcystin on calcium currents

Two other compounds also claimed to inhibit protein phosphatase 1 and 2A are microcystin (MC) and calyculin-A. Therefore, it is presumed that both of them produce similar effects as OA. The drugs were dissolved in DMF, included in the

pipette solution and tested separately.

In Figure 5.9, MC (1 μM) dialysed the cell and changes in the peak Ca current amplitude over a period of time are shown. The recording started 5 min after gaining access to the cell and the maximal current amplitude was seen in 10-17 min (median= 14 min). The peak current amplitude was potentiated, on average, to 2.62±0.08 nA (n= 9) and MC produced apparently slightly stronger enhancement of the peak Ca current than OA. The potentiation of the peak current was significantly larger than in control experiments, $p< 0.001$.

Moreover, this was not the only effect seen in the presence of MC. Fast "run-down" was prevented as in OA cells. The rate of "run-down" was, on average, 7±0.4%, 30 min after obtaining the maximal peak amplitude. That was significantly less than in control cells (with a "run-down" of 17%), but only slightly different than in OA experiments (8%). The difference observed in the effects of MC and OA might be due to the fact that MC is a membrane impermeable substance, while OA is not. Therefore, it could be argued that the presence of MC in the cells was at the concentration applied, whereas OA could slowly diffuse out of the cell. The two substances produced basically the same effect, only MC was slightly more potent.

Apparently, the same phenomenon was also observed by Frace and Hartzell (1993). They reported that MC was more efficacious, producing a maximum Ca current density nearly twice that of OA in frog cardiomyocytes. The group suggested that because OA is unstable in solution, the action of OA could be blocked by its own products of degradation.

In the Figure 5.9, an application and the effect of 8-OH DPAT on the peak Ca current are also shown. 8-OH DPAT (50 μM) was applied into the external solution via a lowered glass capillary with a broken tip, close to the cell. It produced a very clear effect, that is an inhibition of the Ca current, that peaked, on average, at 1.49±0.08 nA.

Following a prepulse application, the inhibition of the peak Ca current was partially relieved, as in OA dialysed neurones. With the prepulse, the current peaked,

on average, at 1.70±0.08 nA. That result followed the pattern observed in OA cells, suggesting again that OA and MC modulate the peak Ca current via the same pathway.

Figure 5.10 shows current and voltage traces obtained in the same DR neurone. The peak Ca current (A, see Figure 5.9), evoked by a voltage step from V_H -100 mV to -10 mV for 150 ms in the presence of MC, was potentiated in addition of a prepulse (B) to 106.3% of control MC current. The second application of the prepulse produced the same effect (data not shown).

An application of 8-OH DPAT (50 μM) into the bath solution, i.e. to the vicinity of the cell, reduced the peak Ca current even in the presence of MC, as described earlier for okadaic acid. The current and voltage traces are shown in Figure 5.11. The peak Ca current elicited in the presence of MC (1, see Figure 5.9) was depressed by an application of 8-OH DPAT (2) by 38.2%. In addition of a prepulse (3), in the continued presence of 8-OH DPAT, the activation kinetics was fully restored, but the inhibition of the peak current was only partially relieved.

Effect of calyculin-A on calcium currents

Calyculin-A is another inhibitor of PP1 and PP2A and a member of OA family of compounds. An effect of the drug on the peak Ca current amplitude is shown in time-dependent fashion in Figure 5.12. Calyculin-A, included in a recording pipette, enhanced the peak amplitude of Ca current, on average, to 2.50±0.04 nA, n= 7. The maximal peak current stabilised on average in 8-16 min from the start of the recording and the compound also slowed the "run-down"; the inset shows that 30 min

later the peak current was 8±0.6% smaller. During the course of recording, a prepulse was intermittently applied and potentiated the peak Ca current to 104% of control. This action of calyculin-A has been observed by other groups (Elmslie et al. 1993).

All three compounds, that are highly specific protein phosphatase 1 and 2A blockers, OA, MC and calyculin A, but not 1-NO, which is an inactive analogue of OA, increased the peak Ca current amplitude and prevented rapid "run-down" to a certain degree. However, these compounds were unable to relieve the inhibition of the HVA Ca current following an application of 8-OH DPAT.

Effect of GTP-γ-S in the presence of okadaic acid

GTP-γ-S is a non-hydrolysable analogue that fully and irreversibly activates G-protein. In the control experiments it exhibited the effect on the peak Ca current that was similar to the one produced by 8-OH DPAT. The two main observations were: decrease of the peak Ca current amplitude and slowing of the activation kinetics.

Here, GTP-γ-S (200 μM) was included, together with OA (1 μM), in a recording pipette and the peak Ca current amplitude at 20° C is shown in Figure 5.13. In control experiments, i.e. in the absence of OA, GTP-γ-S significantly decreased the peak Ca current, on average, to 1.02±0.04 nA. With an addition of OA, the current peaked significantly higher, at 1.90±0.08 nA, $p< 0.001$, at 20° C. "Run-down" was slower and 30 min later, the peak Ca current decreased by only 10%.

With an increase in temperature, the current peaked at higher amplitudes and "run-down" was much faster. The amplitude of the peak current at 25° C, was on average 2.70±0.14 nA. That was a significant change when compared to the cells perfused

with GTP-γ-S, but not with OA. In those cells, the Ca current peaked at 1.68 nA, $p< 0.001$.

A prepulse potentiated the peak Ca current to 115% of control at 20° C, and the current peaked, on average, at 2.20±0.04 nA. In subsequent applications of the prepulses, the effect was repeatable. As expected, the action was even more pronounced at 25° C; the prepulse enhanced the peak Ca current to 3.20±0.1 nA, n= 3. Full recovery of the peak current occurred, with a return of the temperature to 20° C.

Furthermore, when the current traces were analysed an additional effect was observed. Apparently, the activation time in the presence of GTP-γ-S was slower. The effect on the peak Ca current amplitude and activation kinetics in DR neurones dialysed with both GTP-γ-S and OA is shown in Figure 5.14. The peak current obtained in the presence of GTP-γ-S and OA at 20° C, shows smaller peak current amplitude and slowing of the activation kinetics, compared to control cells. However, with an addition of the prepulse the block was substantially relieved and the activation kinetics fastened. The changes in the current kinetics are described later.

At 25° C, the current peaked at higher amplitudes and the prepulse additionally potentiated the peak current, see Figure 5.15. In Figure 5.16 two superimposed traces of the peak Ca current evoked by voltage pulses at 20° and 25° C respectively, are shown in the absence of a prepulse. For clearer presentation, the inset (B) shows a current trace obtained when the current elicited at 20° C was subtracted from the current evoked at 25° C.

Effect of calcineurin inhibitors

After analysing the effects of PP1 and PP2A inhibitors on modulation of the peak Ca current, obviously the next step was to examine the involvement of protein phosphatase 2B (PP2B) on Ca channels.

FK 506, an immunosuppressant, has been shown to bind and inhibit calcineurin (Liu et al. 1991; Schwaninger et al. 1993). Its effect depends on the presence of Ca^{2+} and FK-binding protein (FKBP). In the present experiments, FK 506 had no effect on the peak Ca current in the absence of FKBP (data not shown). Figure 5.17 shows a period of time in which the peak Ca current was elicited with voltage pulses from V_H -100 mV to -10 mV, every 20 s. FK 506 (0.01 μM) was dissolved in DMF and together with FKBP-12 (1 μM) included in the recording pipette. The peak Ca current amplitude slowly increased in size and the current was potentiated to 108% of control, in the presence of FK 506 and FKBP, n= 3. One other observation was, that, unlike in OA cells, "run-down" was not significantly reduced, and the peak Ca current was 14% smaller 30 min later, similar to control experiments (17%). However, it was important to examine whether calcineurin mediated 8-OH DPAT inhibition of the peak Ca current. When 50 μM of 8-OH DPAT was applied into the external solution close to the neurone, see inset, the peak current was inhibited, on average, by 27%, as in control and OA dialysed dorsal raphé neurones. Therefore, it appears that there is no direct link between 8-OH DPAT and protein phosphatase 2B action on Ca channel currents.

Effect of forskolin and protein kinase inhibition

Forskolin is recognised as a stimulator of intracellular cAMP (Laurenza et al. 1989;

Dolphin, 1992; Hartzell & Budnitz, 1992). A proposed hypothesis is that 5-HT causes an increase in intracellular cAMP and possibly activates cAMP-dependent protein kinase for its action on DR neurones. So, in the presence of forskolin it was to expect that no further modulation, in addition of 8-OH DPAT, would occur.

Forskolin (10 μM) was dissolved in an anhydrous DMF and included in the pipette solution. Figure 5.18 shows changes of the peak Ca current amplitude over time during one representative experiment. In the presence of forskolin, the peak Ca current was only slightly potentiated and "run-down" was 12%, 30 min later, n= 3. Application of 8-OH DPAT (50 μM) into the external solution inhibited the peak Ca current to the same extent as in the absence of forskolin, by 26%, as shown in Figure 5.19 (A). The same observation was reported in DRG neurones (Dolphin, 1991b), where baclofen still inhibited Ca current in the presence of forskolin. Further on, with the inclusion of both forskolin and OA (1 μM) in the recording pipette, the peak Ca current was additionally potentiated but the inhibition of the peak current by the 5-HT_{1A} agonist (B) was not abolished, n= 3.

Another compound, that is specific in blocking protein kinase A and C, H-7, was tested in the same fashion. With H-7 in the recording pipette, 8-OH DPAT was applied and the peak current was inhibited, on average, by 31% (C), as in control DR neurones. Staurosporine is a potent, non-selective, protein kinase inhibitor (Herbert et al. 1990). It has been shown that the drug markedly inhibited protein kinase C in rat brain (Tamaoki et al. 1986). A similar potency was reported for its inhibition of cAMP-dependent protein kinase from bovine heart. When staurosporine (1 μM) dialysed DR neurones, the inhibition of the peak Ca current by the 5-HT_{1A} agonist was not prevented (n= 2, data not shown). The same result was reported by Yakel (1992) in dissociated neurones from the snail *Helix aspersa.*

It seems that a stimulation of cAMP by forskolin modulates Ca channel currents in DR neurones. However, it is clear that OA and 8-OH DPAT do not use the same modulatory pathway as forskolin to exercise their action on the HVA Ca current.

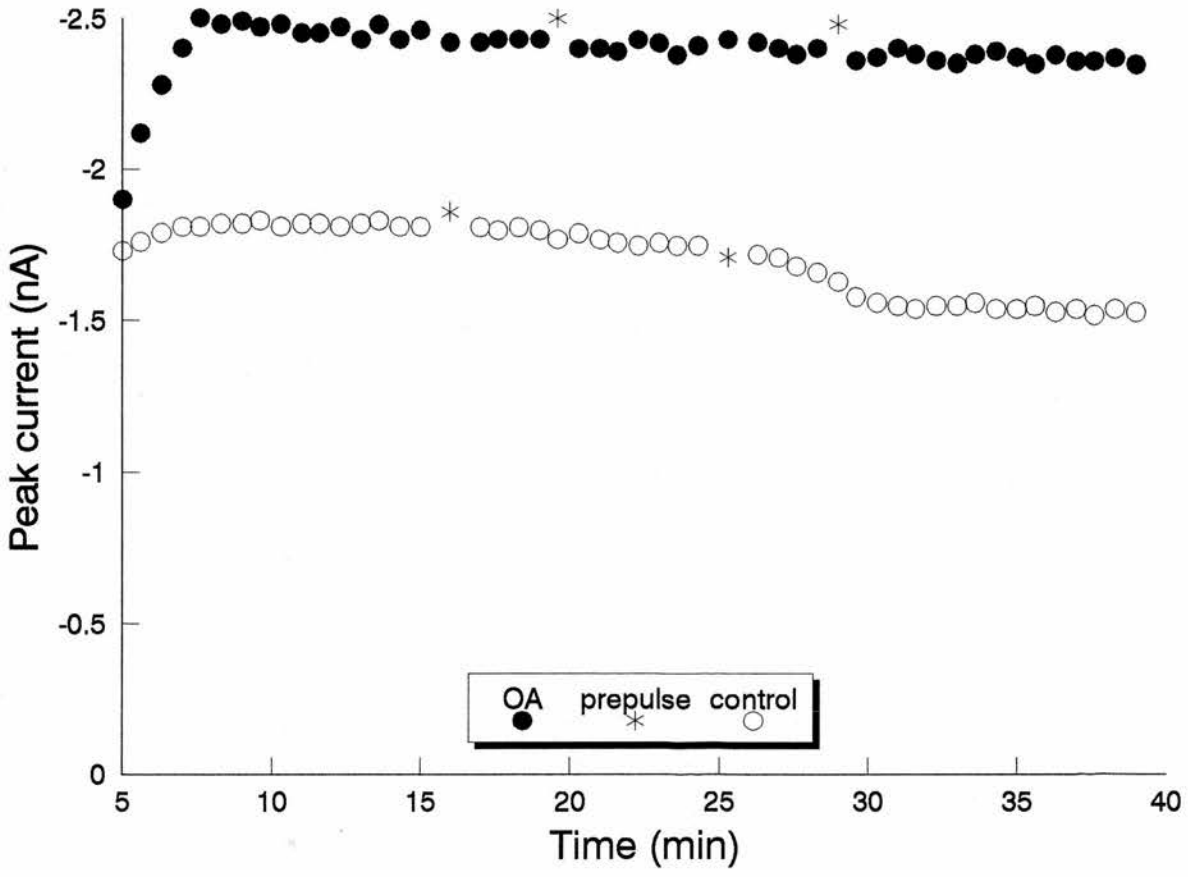

Figure 5.1: Enhancement of the peak calcium current and attenuation of "run-down" by okadaic acid. Peak amplitude (●) of Ca current evoked with voltage steps from V_H -100 mV to -10 mV for 150 ms at 20° C is plotted against time. In the presence of OA the peak current amplitude was maximal, on average, in 13.6 min (n= 45), rather later than in control neurones (mean of 7.6 min). The peak current was potentiated to 139%, compared to control experiments ($p< 0.01$). "Run-down" was significantly reduced in the presence of OA, and the peak current decreased by 8% over 30 min (compared to 17% in the absence of OA, ○). Application of a prepulse (✱) additionally potentiated the peak current. OA (1 μM) was dissolved in DMF and included in the recording pipette. Standard internal and external solutions were used. Currents are leak and capacity subtracted. Cells 1105932 and 0902932.

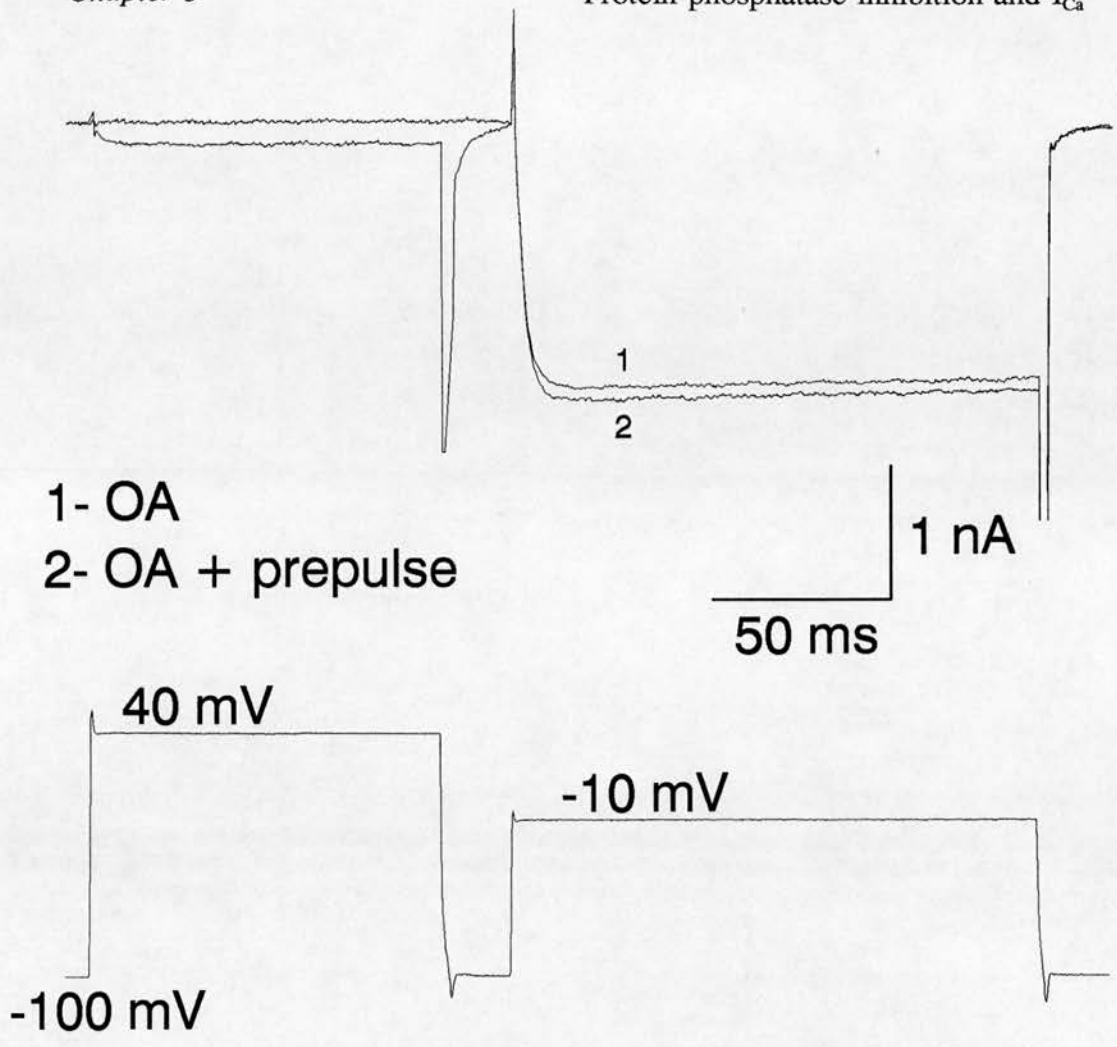

Figure 5.2: Okadaic acid produced an increase in the peak calcium current that was additionally potentiated by a prepulse. In DR neurones dialysed with 1 μM OA, the current peaked, on average, at 2.53 nA, n= 45, significantly higher than in control cells (1.82 nA, $p< 0.01$). In addition of a prepulse, further enhancement of the peak current was observed, and the current amplitude was 2.61 nA, n= 21. Superimposed current traces of the peak current elicited in the presence of OA (1) and in addition of a prepulse (2), evoked by voltage pulses (lower panel) are shown. Currents are leak and capacity subtracted. Standard internal and external solutions were used. Cell 1105931.

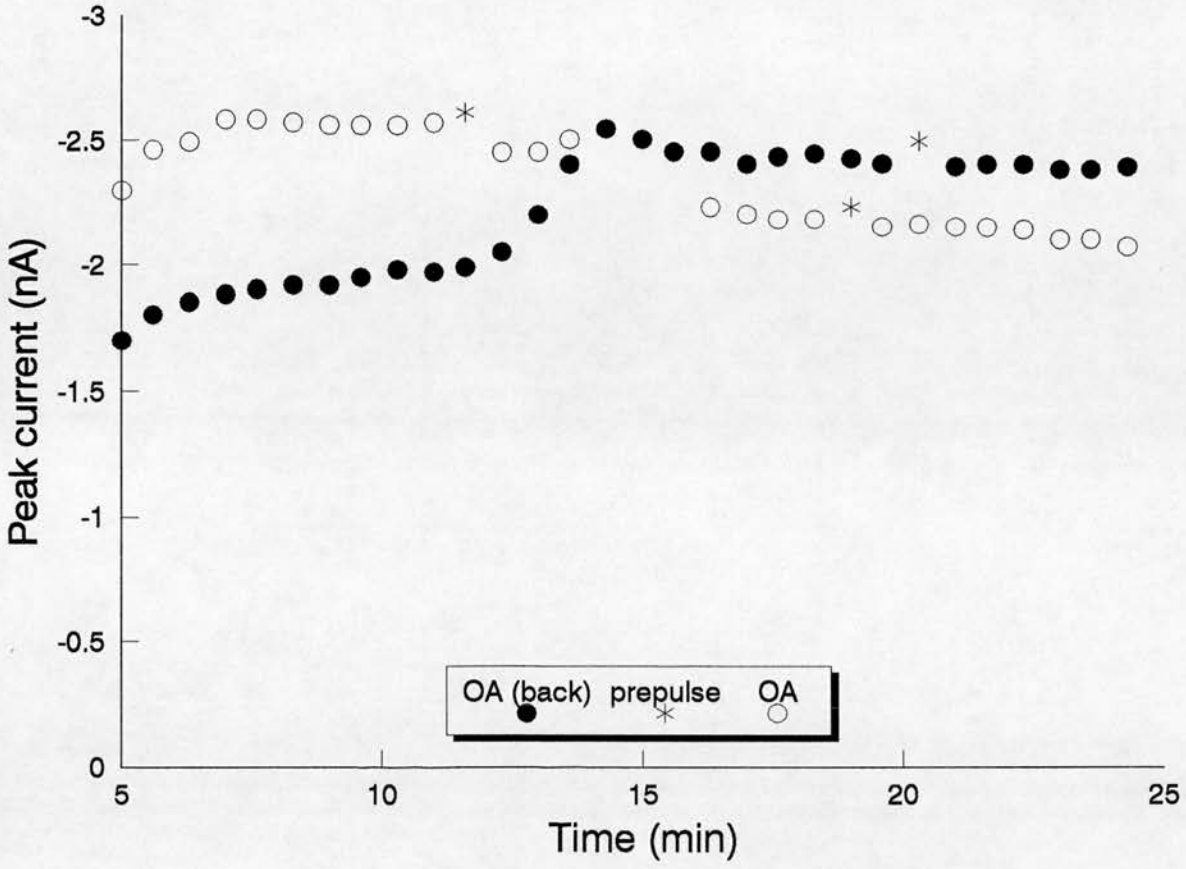

Figure 5.3: "Back filling" delayed potentiation of the peak calcium current by okadaic acid. OA (1 μM) was included only at the back of the recording pipette, with the tip filled by a standard recording solution. Diffusion of OA (●) into the DR neurone was slower and maximal peak amplitude of the current was observed in 16 min (n= 3), rather later than in control OA cells (13.6 min, ○). However, once the compound dialysed the neurone, it potentiated the current and similar rate of "run-down" as in control OA cells was recorded (8%). A prepulse (✱) additionally enhanced the peak current. Currents are leak and capacity subtracted. Standard internal and external solutions were used. Cells 0906931 and 1105934.

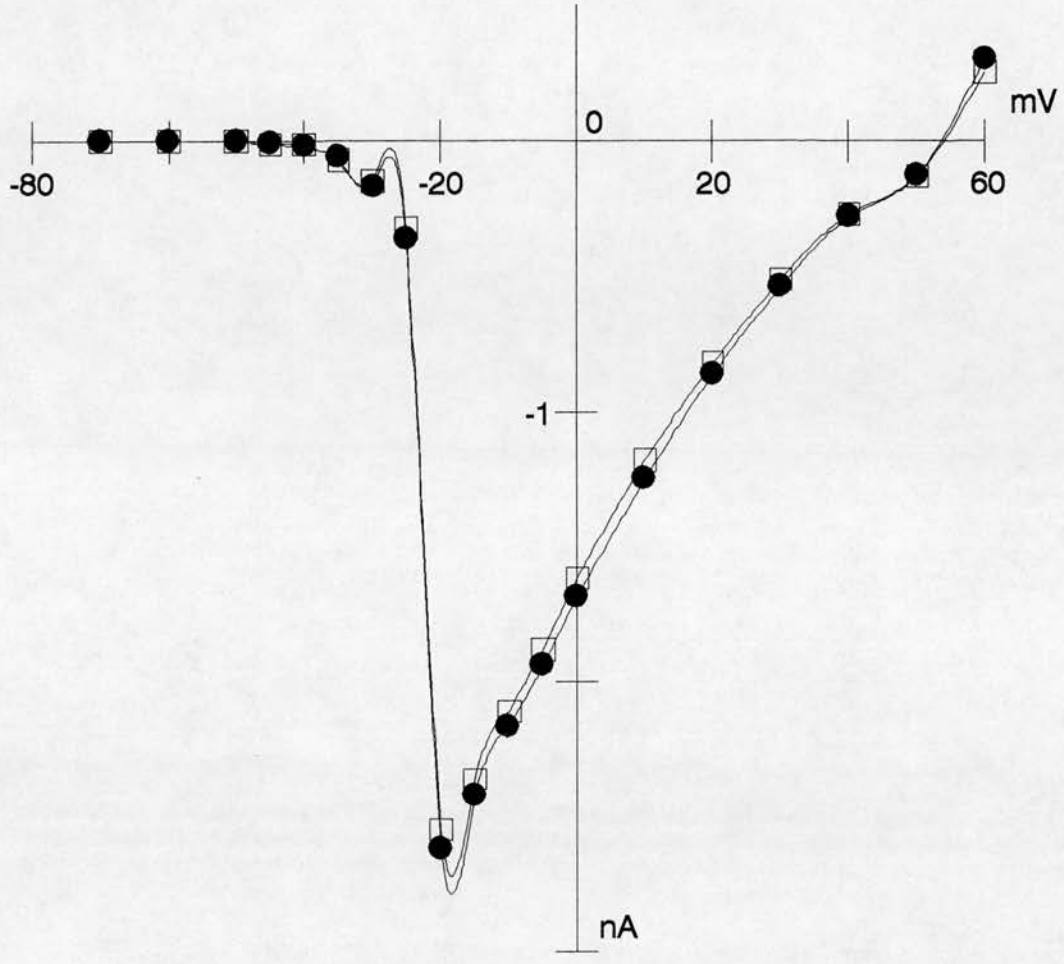

Figure 5.4: Current-voltage relationship of calcium currents recorded in a typical DR neurone dialysed with okadaic acid at 20°C. Ca currents were evoked by voltage jumps from a holding potential of -100 mV to a series of depolarised test potentials for 150 ms. The relation between the test potentials and transient (●) and slowly decaying (❑) components of the current is shown. The activation threshold was at -40 mV, the peak current was observed at around -15 mV and reversal potential at 50 mV. Note that there was almost no inactivation of the current present. OA (1 μM) was included in the internal solution. Currents are leak and capacity subtracted. The curve was fitted using a cubic spline routine. Standard internal and external solutions were used. Cell 2705931.

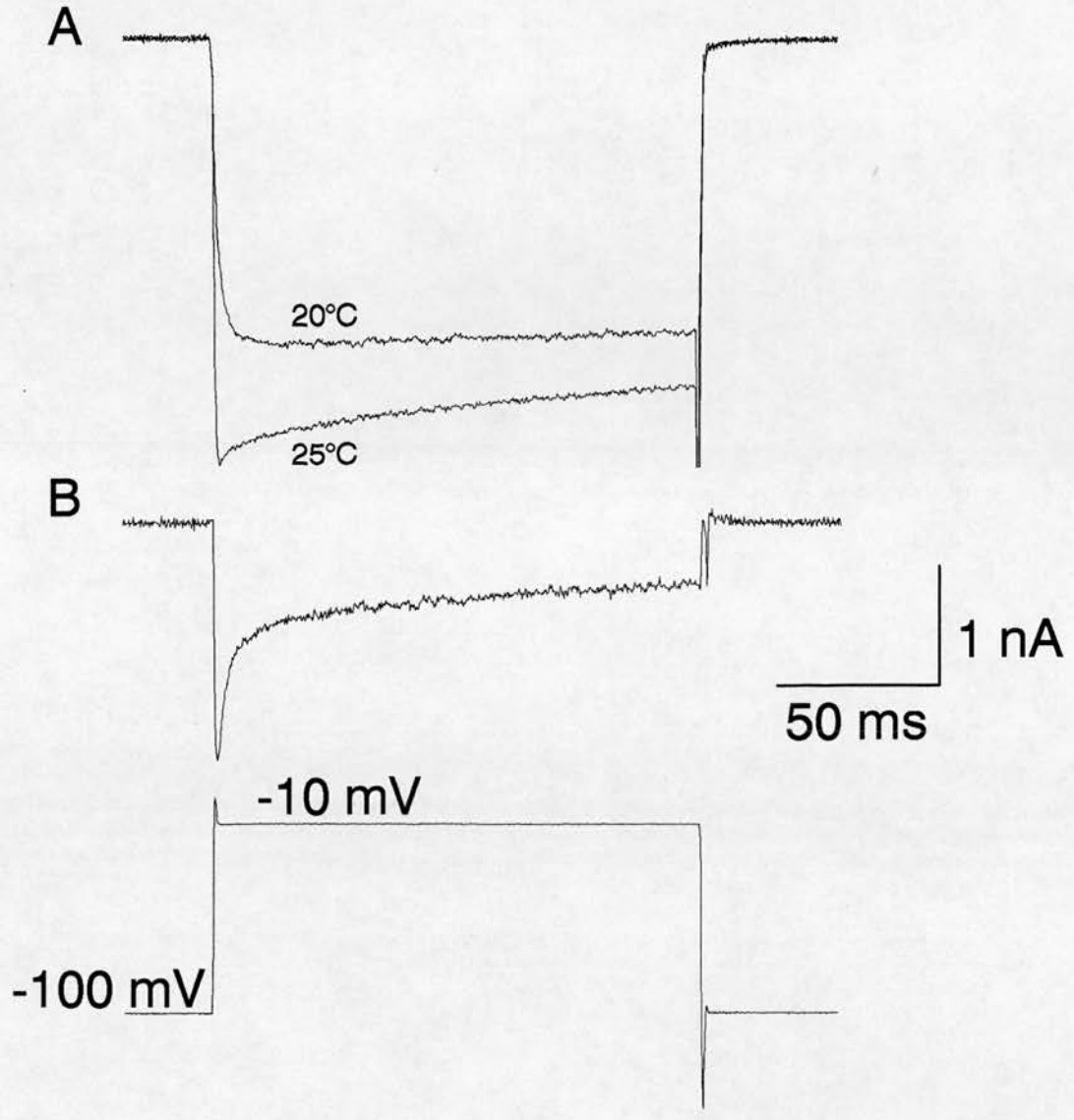

Figure 5.5: Temperature-dependency of the peak calcium current in the presence of okadaic acid. Peak Ca current evoked in a typical DR neurone with standard voltage pulses at 20° and 25° C, respectively, are shown. With an increase in temperature the current peaked at higher amplitude, activation was speeded up and inactivation was stronger, see text. Ca currents peaked, on average, at 2.53 nA (n= 45) and 3.23 nA (n= 5), at 20° and 25° C, respectively (A). Both transient and decaying components were affected by an increase in temperature, as shown in the inset (B). OA (1 μM) was included in the recording pipette. Currents are leak and capacity subtracted. Standard internal and external solutions were used. Cell 1606932.

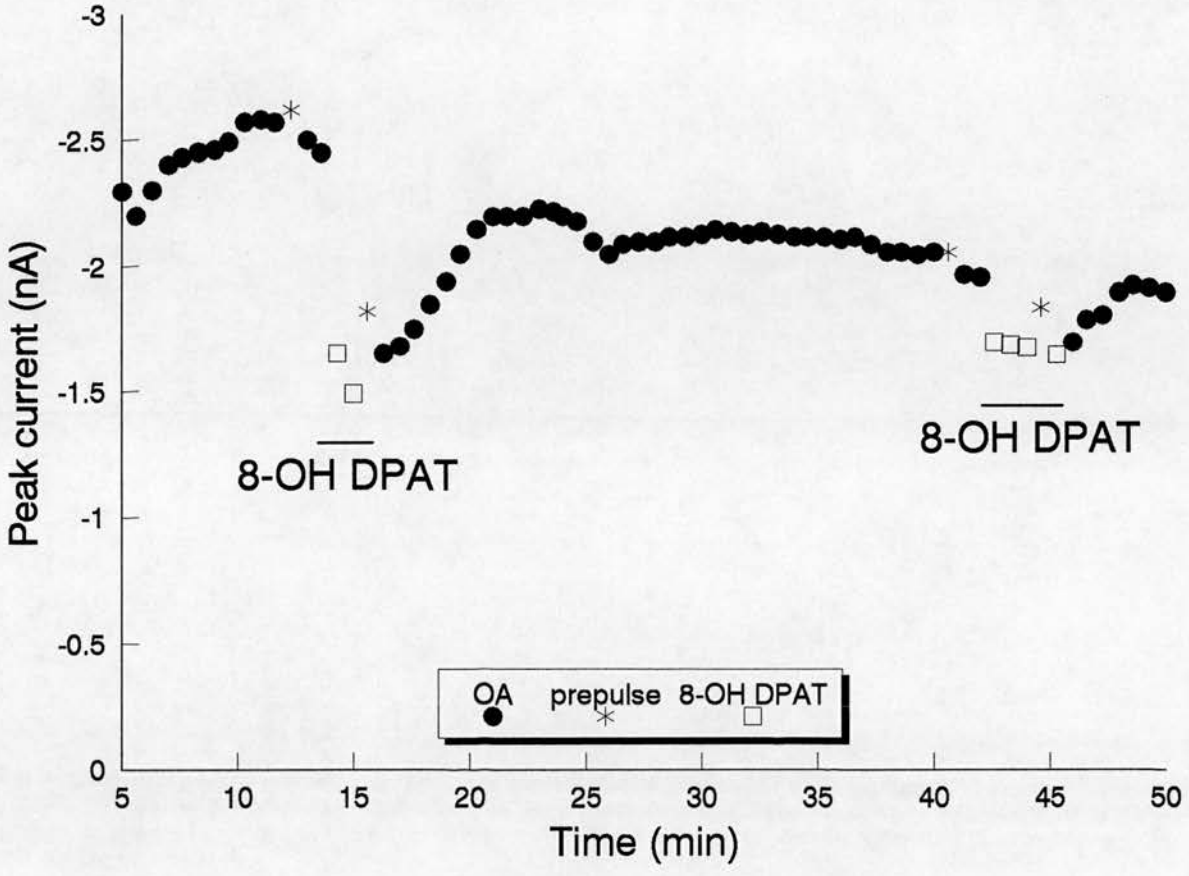

Figure 5.6: Time course of the reduction of the peak calcium current by 8-OH DPAT in OA dialysed cells. Peak Ca current (●) was evoked over a period of time in a typical DR neurone. The current stabilised and 50 μM 8-OH DPAT (❑) was applied (horizontal bar) externally via a broken tip glass pipette close to the cell. The drug inhibited the peak current, on average, by 31%, n= 16 and the effect was repeatable and fully reversible following wash-out of 8-OH DPAT. As in control cells, prepulse (✱) significantly relieved the inhibition of the peak Ca current. Currents are leak and capacity subtracted. OA (1 μM) was included in the internal solution. Standard internal and external solutions were used. Cell 1702942.

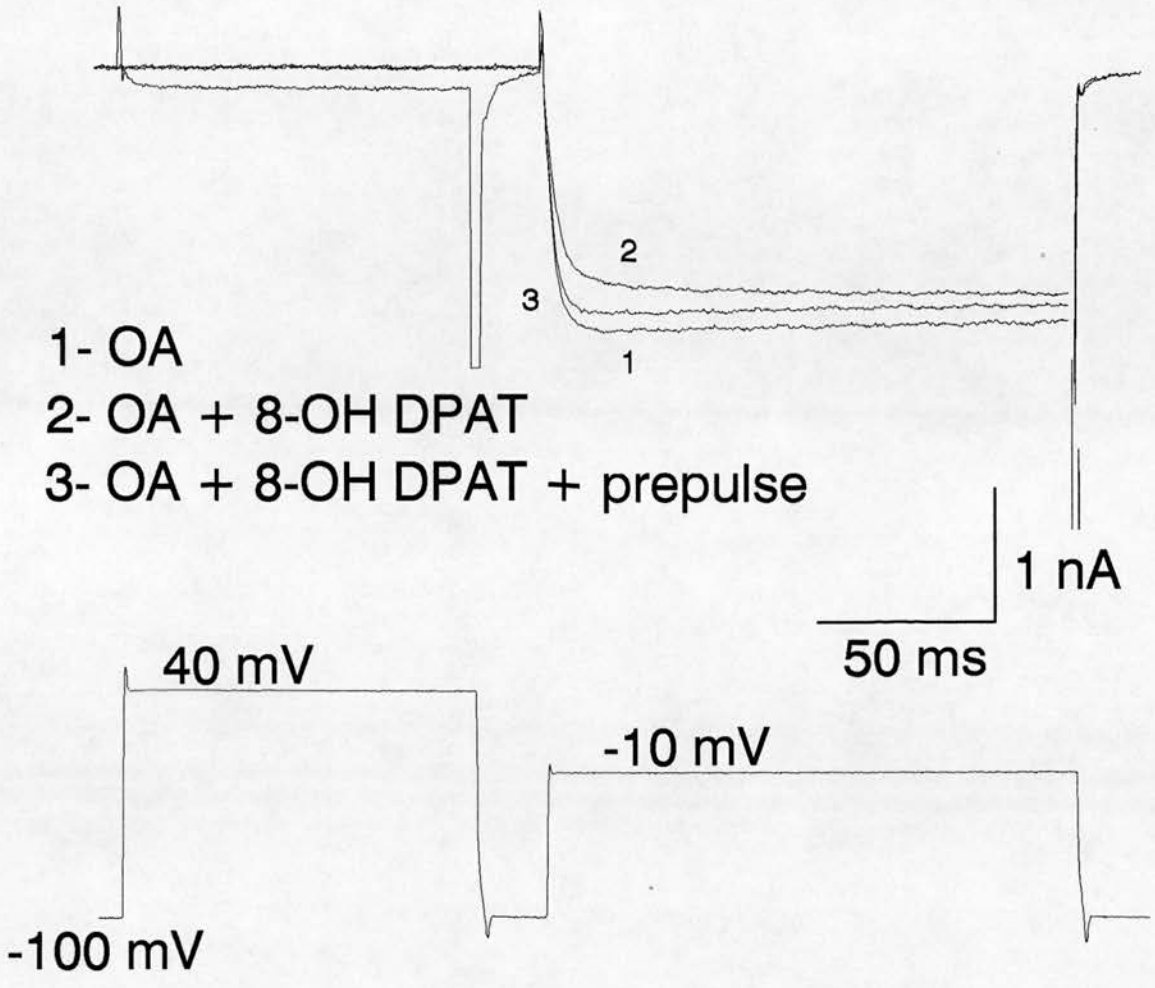

Figure 5.7: Okadaic acid had no effect on the inhibition of the peak calcium current by 8-OH DPAT. In OA (1 μM, 1) dialysed cells Ca current peaked at 2.53 nA (n= 45) and following bath application of 8-OH DPAT (50 μM, 2) significantly decreased and peaked, on average, at 1.56 nA (n= 16, $p< 0.001$). A prepulse (3), applied in the presence of both 8-OH DPAT and OA, significantly relieved the inhibition by 45% and the mean peak current was 1.82 nA. Voltage pulses are shown in the lower panel. OA was included in the recording pipette and standard internal and external solutions were used. Currents are leak and capacity subtracted. Cell 2402941.

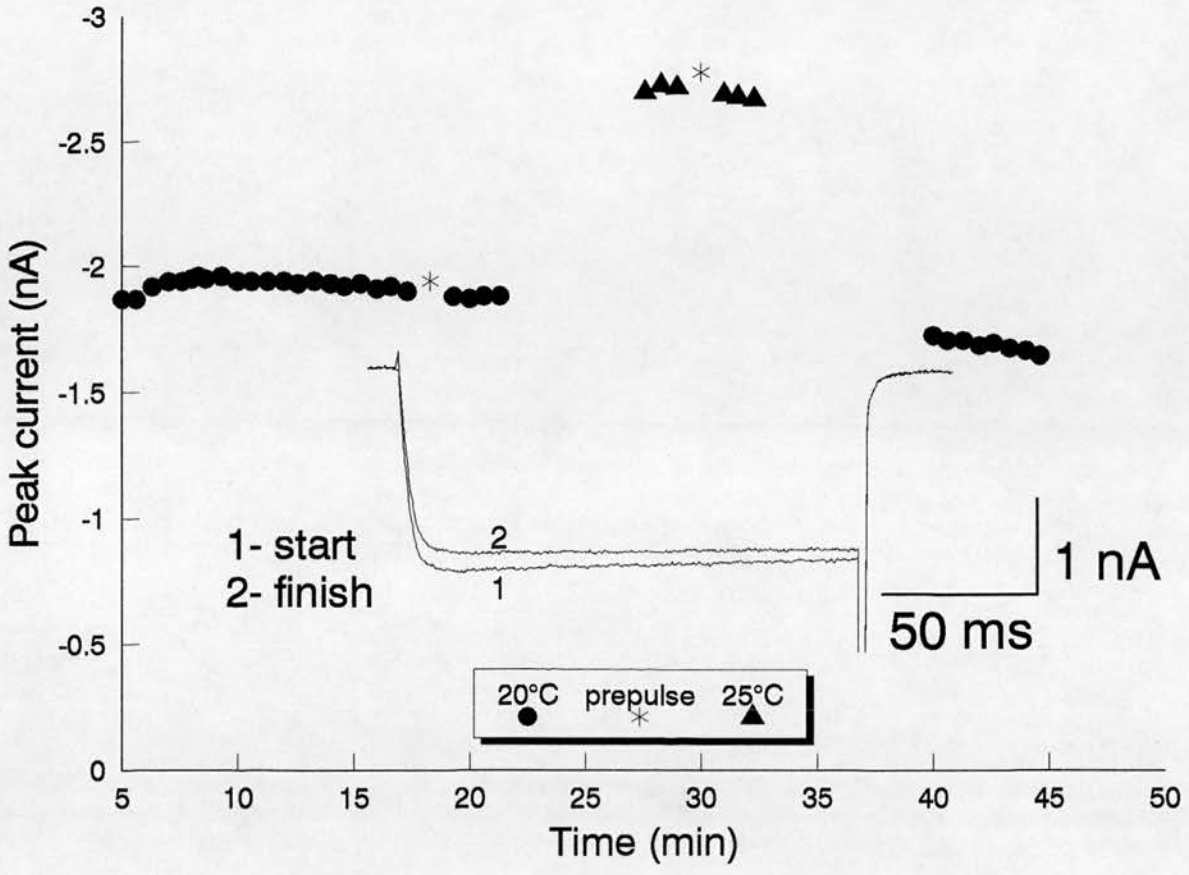

Figure 5.8: 1-norokadaone, an inactive analogue of okadaic acid, had no effect on the peak calcium current. 1-NO (1 μM, ●) was included in the recording pipette and the peak current was plotted against time. Maximal current amplitude was recorded in 10.3 min, rather earlier than in control OA cells (13.6 min). "Run-down" was not prevented and 30 min later, the peak current was 16% smaller, at 20° C (see inset). With an increase in temperature to 25° C, the current (▲) was reversibly potentiated and peaked, on average, at 2.73 nA, compared to 3.23 nA observed in OA dialysed neurones. Prepulse (✱) produced a weak enhancement of the peak current, to 102% of control. Currents are leak and capacity subtracted. Standard internal and external solutions were used. Cells 0507942 and 0707941 (inset).

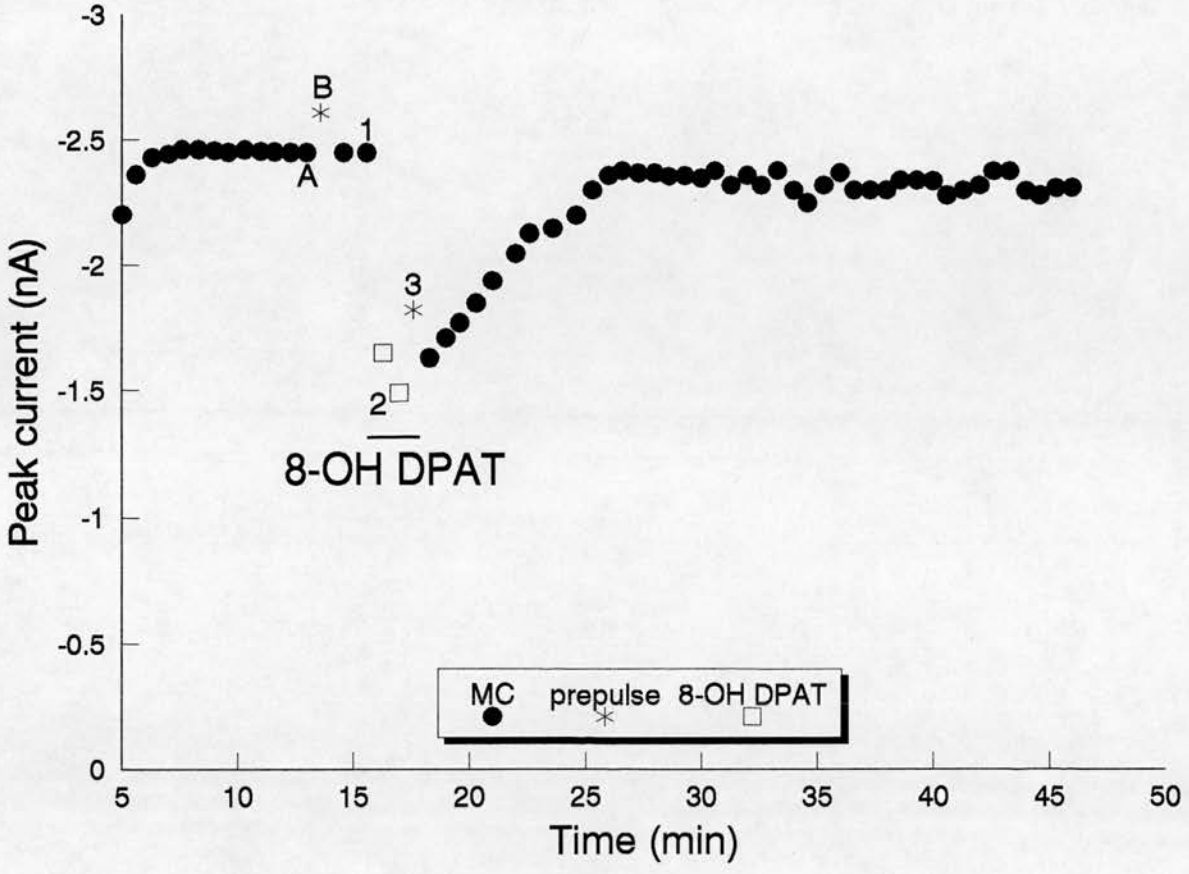

Figure 5.9: "Run-down" and time course of 8-OH DPAT-induced inhibition of the peak calcium current in the presence of microcystin. The recording started 5 min after obtaining a whole-cell mode and the maximal current amplitude was observed, on average, in 14 min. The peak amplitude was potentiated to 2.62 nA (n= 9), significantly higher than in control cells (1.82 nA, $p< 0.001$). Fast "run-down" was prevented and the peak current was decreased by 7%, 30 min later. 8-OH DPAT (horizontal bar) inhibited the peak current, on average, by 38%. The effect was partially relieved by a prepulse (✱). MC (1 μM, ●) was dissolved in DMF and included in the internal solution and 8-OH DPAT (50 μM, ❑) was applied externally. Currents are leak and capacity subtracted. Standard internal and external solutions were used. Cell 1304931.

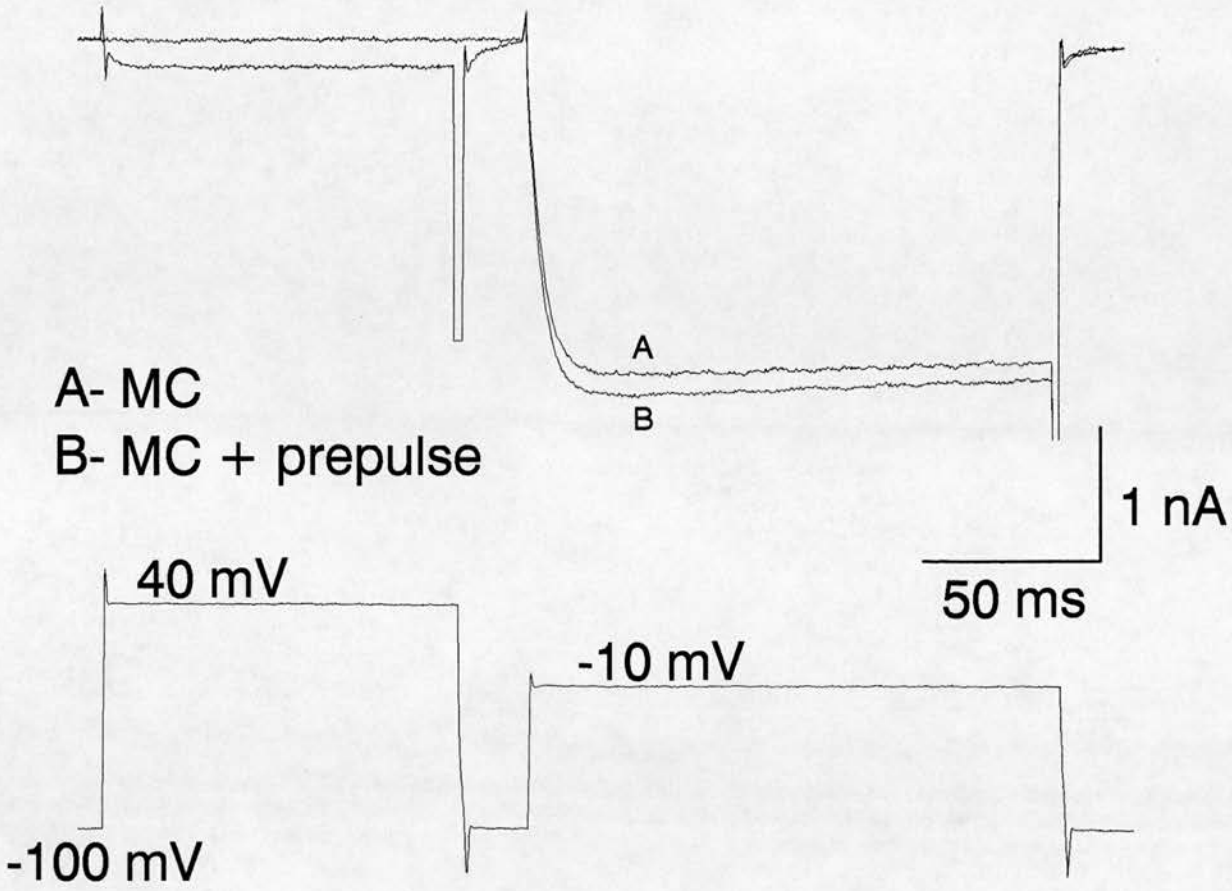

Figure 5.10: Enhancement of the peak calcium current by microcystin was additionally potentiated by a prepulse. MC (1 μM) was included in the recording pipette and strongly potentiated the peak current, on average, to 2.62 nA (A), compared to 1.82 nA in control and 2.53 nA in OA dialysed cells. Application of a prepulse (B) in the presence of MC additionally increased the current by 6%. Currents are leak and capacity subtracted. Standard voltage pulses (lower panel) and standard internal and external solutions were used. Cell 1304931.

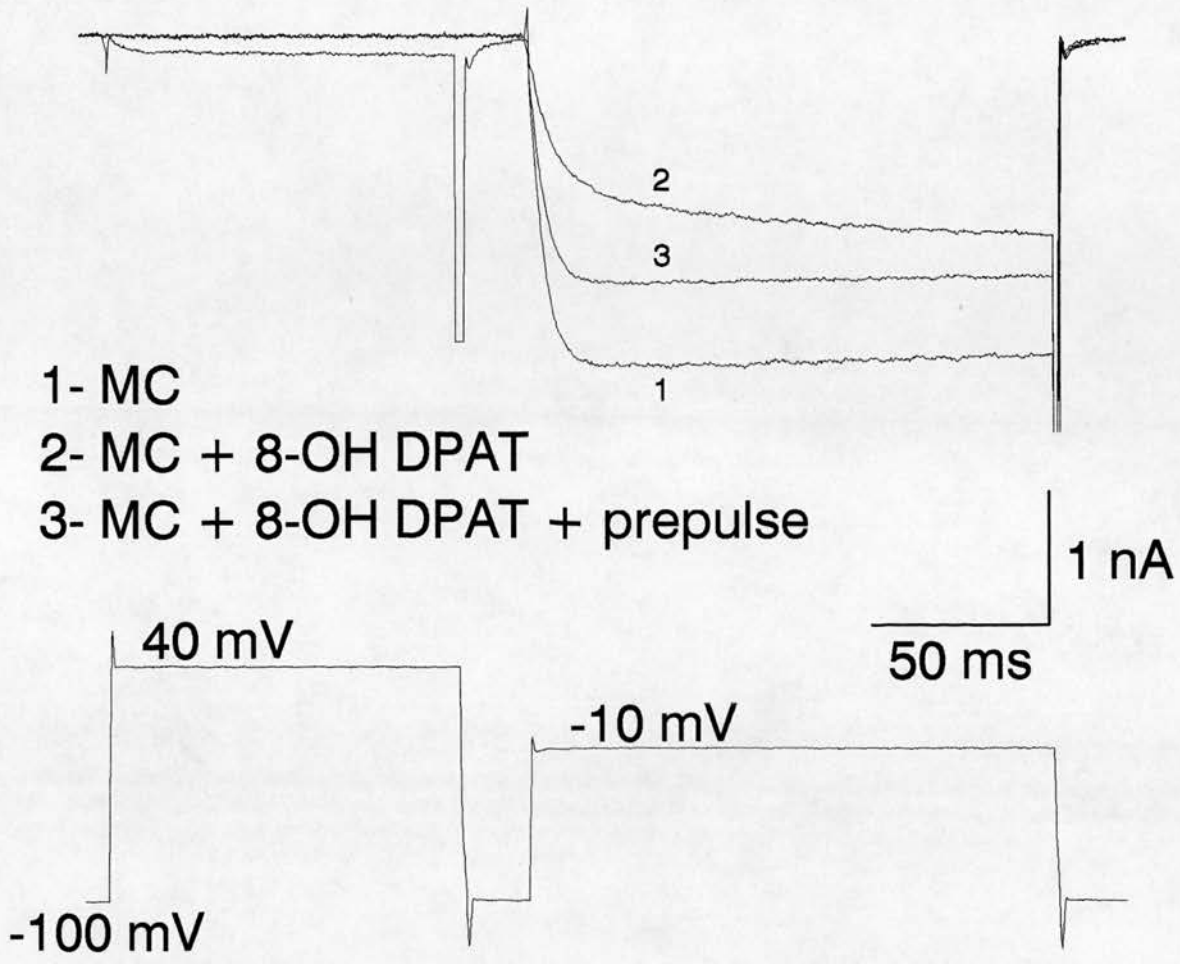

Figure 5.11: Microcystin had no effect on the inhibition of the peak calcium current by 8-OH DPAT. Ca current evoked in the presence of MC peaked, on average, at 2.62 nA (1) and in addition of 8-OH DPAT the current was inhibited by 38%, and peaked, on average, at 1.49 nA (2). Application of a prepulse (3), in a continuous presence of MC and 8-OH DPAT substantially relieved the inhibition, similar as in control and OA cells. Superimposed peak current traces are shown, evoked with standard voltage jumps (lower panel). MC (1 μM) was included in the recording solution and 8-OH DPAT (50 μM) was applied extracellularly. Currents are leak and capacity subtracted. Standard internal and external solutions were used. Cell 1304931.

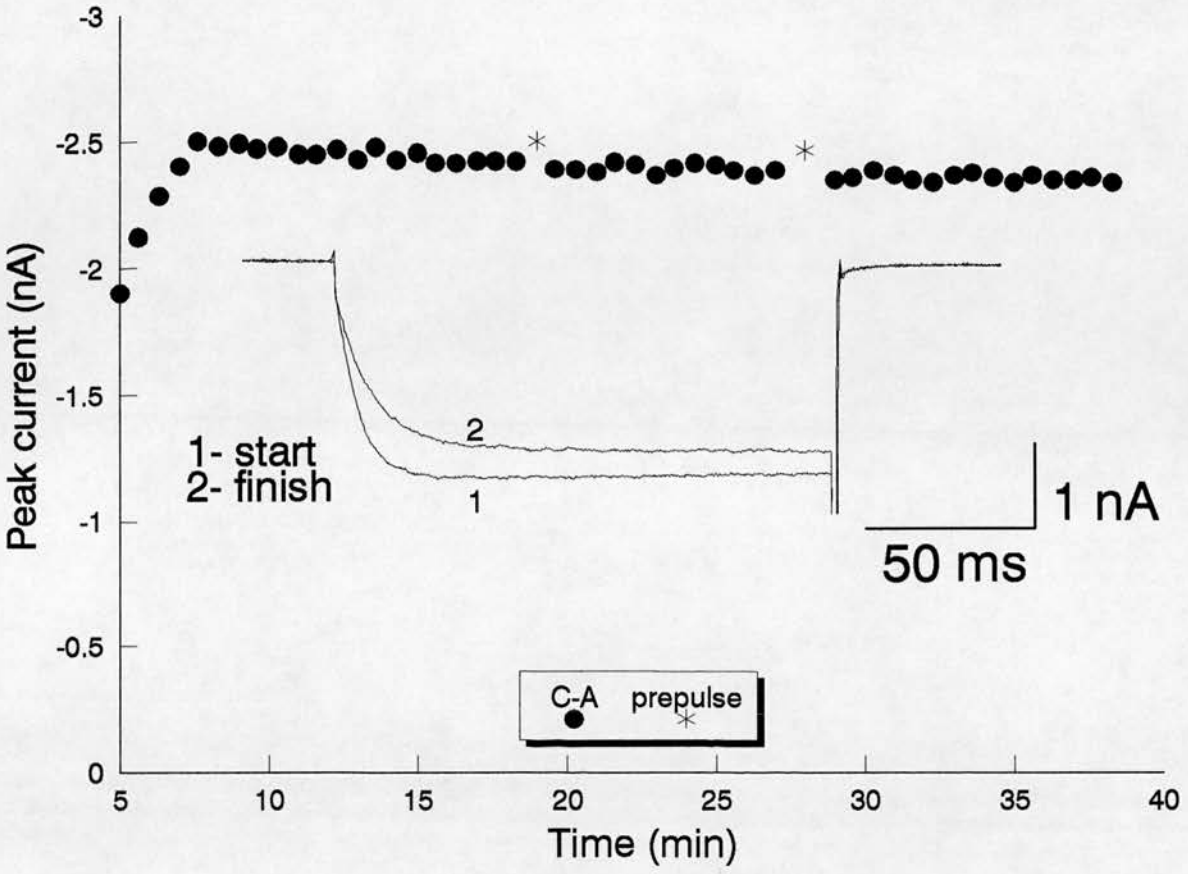

Figure 5.12: Effect of calyculin-A on the peak calcium current amplitude and "run-down". Peak Ca current evoked over a period of time in the presence of calyculin-A (●) is shown. Maximal current amplitude was observed, on average, in 13.5 min and the peak amplitude was potentiated, on average, to 2.50 nA, n= 7. "Run-down" was significantly slower than in control cells, and the current decreased by 8%, 30 min later. Application of a prepulse (✱) additionally enhanced the current to 104% of control. Inset shows two superimposed peak Ca current traces obtained at the start (1) and the end (2) of the recording. Calyculin-A (1 μM) was dissolved in DMF and included in the recording pipette. Currents are leak and capacity subtracted. Standard internal and external solutions were used. Cell 0405941.

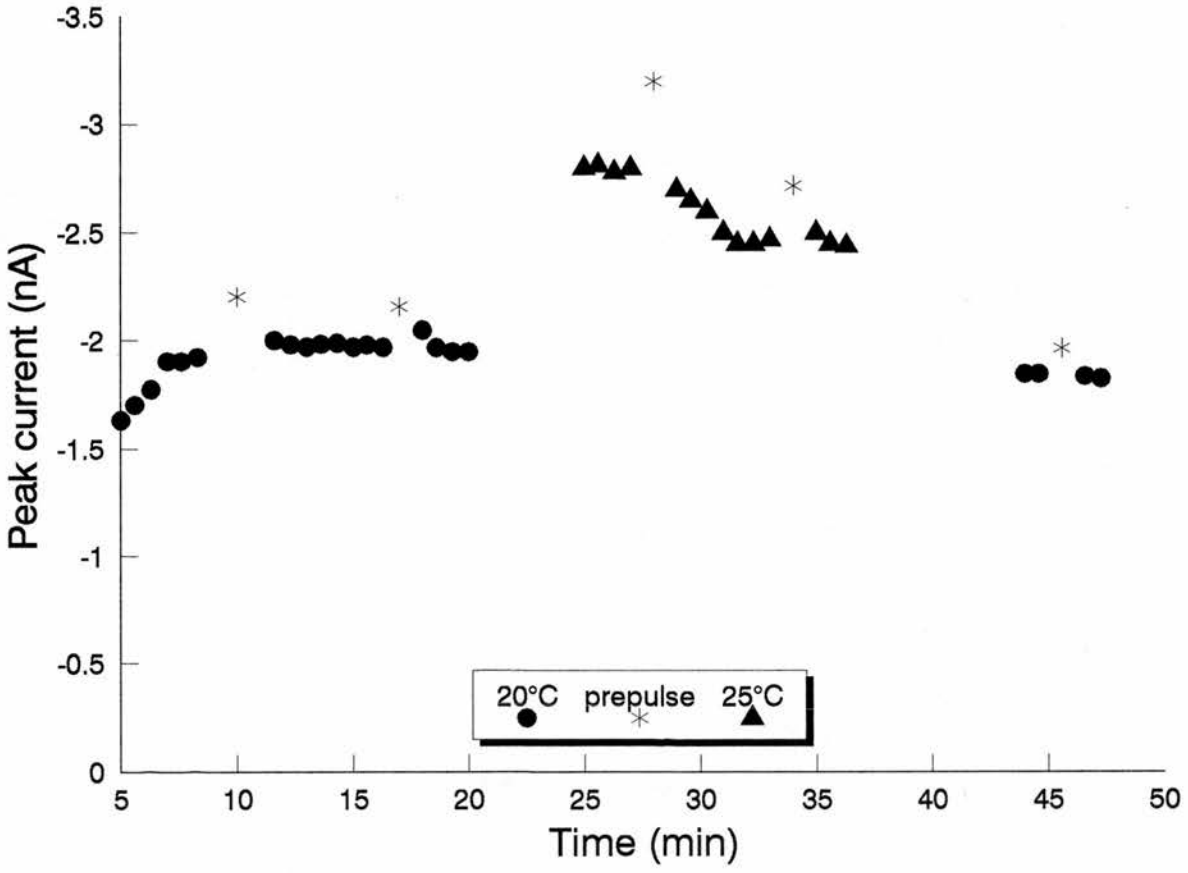

Figure 5.13: Time course of GTP-γ-S effect in DR neurones dialysed with okadaic acid. Peak Ca current at 20° C (●) and 25° C (▲) is plotted against time. With the addition of GTP-γ-S, the current in the presence of OA peaked, on average, at 1.90 nA, significantly higher than in the absence of OA (mean of 1.02 nA, $p < 0.001$), but lower than in control OA cells (2.53 nA). "Run-down" was reduced and the peak current was 10% smaller, 30 min later. The current was temperature dependent and at 25° C peaked, on average, at 2.70 nA. Application of a prepulse (✱) enhanced the peak current to 115% of control. GTP-γ-S (200 μM) and OA (1 μM) were included in the recording solution. Currents are leak and capacity subtracted. Standard internal and external solutions were used. Cell 1805942.

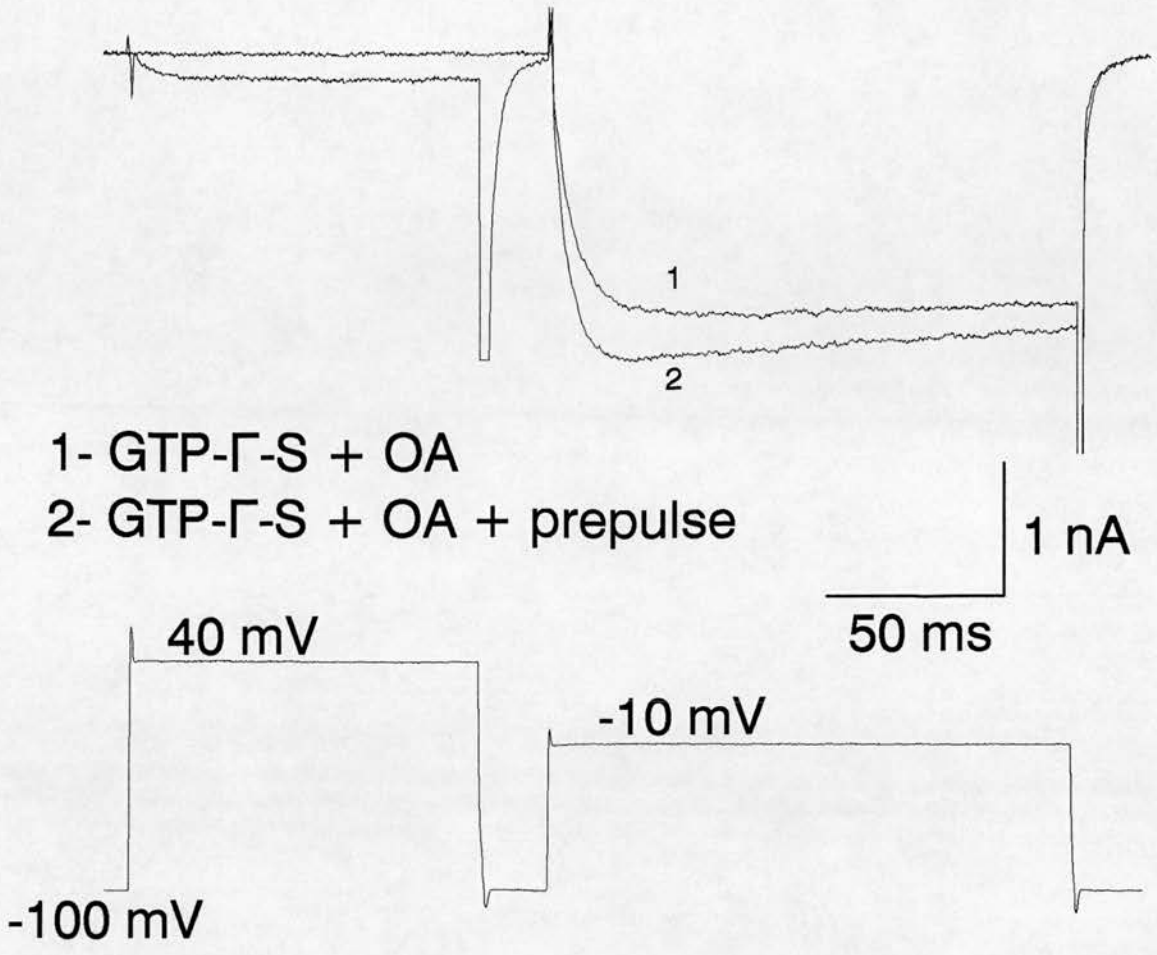

Figure 5.14: Effect of GTP-γ-S in the presence of okadaic acid at 20°C. Peak Ca current traces are superimposed in the presence of GTP-γ-S and OA (1) and in addition of a prepulse (2). At 20° C Ca current was evoked using standard voltage pulses (lower panel) and peaked, on average, at 1.90 nA. Application of a prepulse partially relieved the inhibition of the peak current by 14%. With the prepulse Ca current peaked, on average, at 2.20 nA. GTP-γ-S (200 μM) and OA (1 μM) were included in the internal solution. Currents are leak and capacity subtracted. Standard internal and external solutions were used. Cell 2605941.

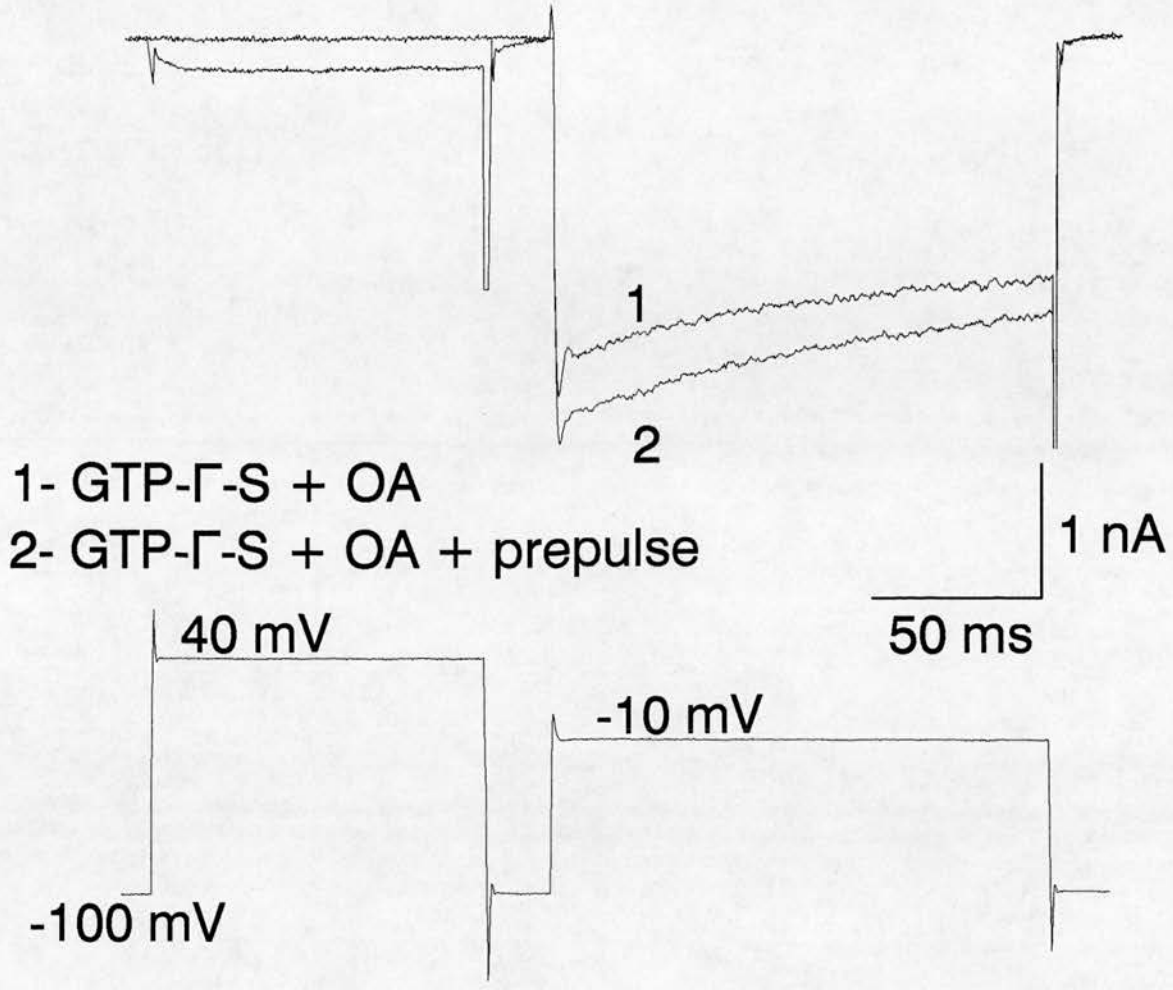

Figure 5.15: Effect of GTP-γ-S in the presence of okadaic acid at 25° C. At 25° C, the current peaked significantly higher, at 2.70 nA, than at 20° C (1.90, $p < 0.001$), but lower than in control OA cells at 25° C (3.23 nA). Application of a prepulse partially relieved the GTP-γ-S-induced inhibition by 16% and potentiated the peak current, on average, to 3.20 nA. The peak current elicited in the presence of both GTP-γ-S and OA (1) and in addition of a prepulse (2) are shown, together with voltage pulses (lower panel). Currents are leak and capacity subtracted. GTP-γ-S (200 μM) and OA (1 μM) were included in the recording pipette. Standard internal and external solutions were used. Cell 2605944.

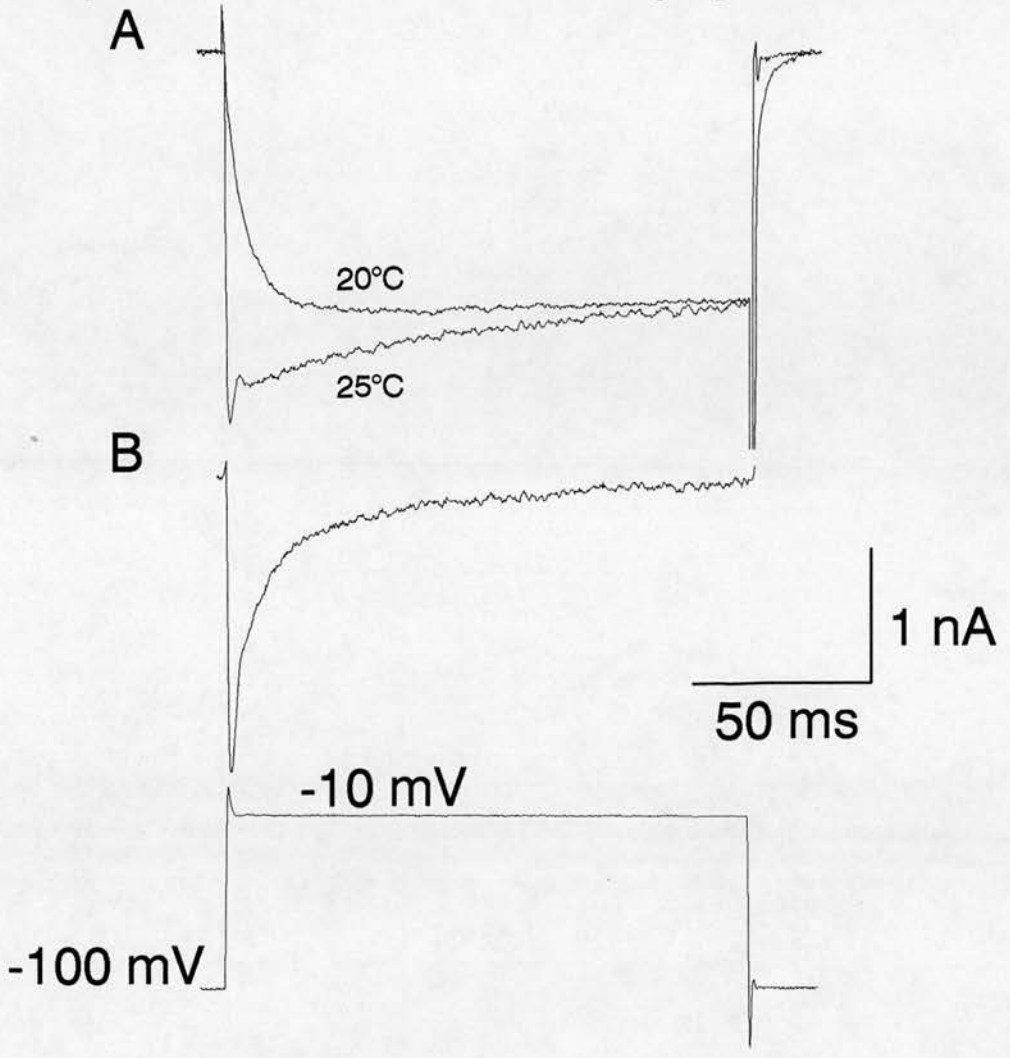

Figure 5.16: A comparison of the peak calcium current evoked in the presence of GTP-γ-S and okadaic acid at 20° and 25°C. In (A) the peak current elicited in the cells dialysed with GTP-γ-S and OA at 20° and 25° C are superimposed, as indicated. The peak current amplitudes were reduced compared to control OA cells, thus mimicking the effect of 8-OH DPAT in DR neurones dialysed with OA. In (B) the inset shows that mainly transient component of the peak current was enhanced in most cells at 25° C. Standard voltage pulses were used (lower panel). GTP-γ-S (200 μM) and OA (1 μM) were included in the recording pipette. Currents are leak and capacity subtracted. Standard internal and external solutions were used. Cell 2605942.

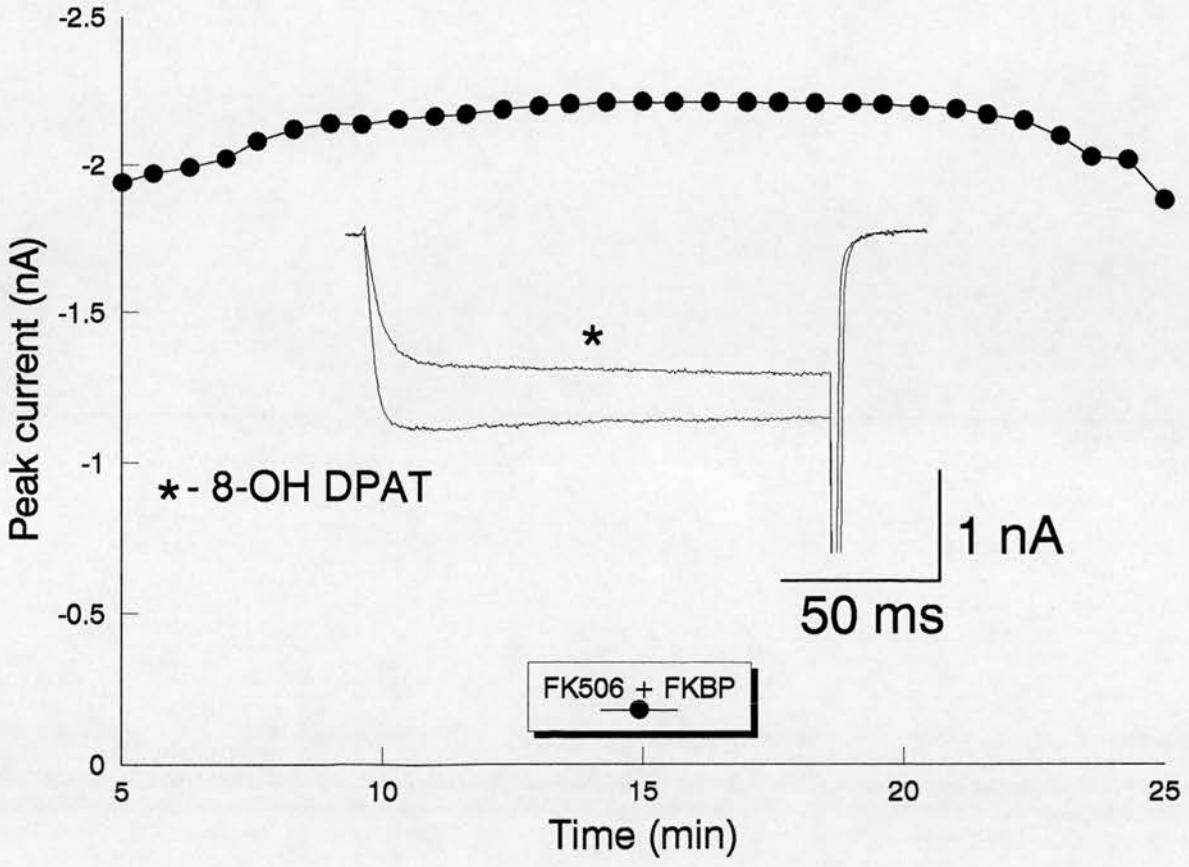

Figure 5.17: FK 506 up-modulated the peak calcium current and had no effect on the 8-OH DPAT action. Peak current amplitude in the presence of FK 506 and FKBP (●) in the recording pipette is plotted against time. The peak current was potentiated to 108% (n= 3), compared to control experiments. "Run-down" was slightly reduced and the peak current decreased by 14% over 30 min. Application of 8-OH DPAT led to a significant, but incomplete reduction of the peak current (26%), similar as in control experiments. The peak current in the presence of FK 506 (0.01 μM) and FKBP (1 μM) and in addition of 8-OH DPAT (50 μM, ✱) is shown in the inset. Currents are leak and capacity subtracted. Standard internal and external solutions were used. Cells 0507941 and 0507943 (inset).

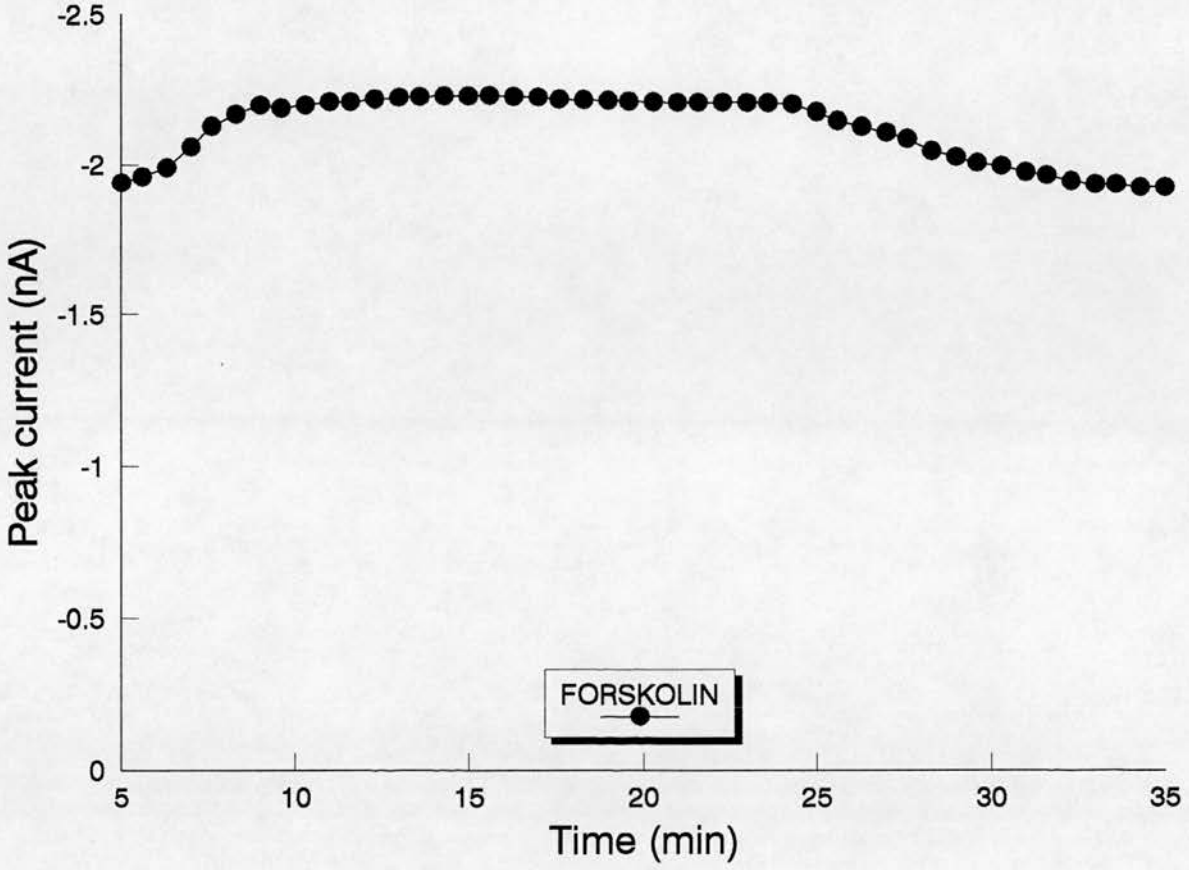

Figure 5.18: Effect of forskolin on the peak calcium current. Forskolin (10 μM, ●) was dissolved in DMF and included in the recording pipette. Peak Ca current was evoked over a period of time with standard voltage jumps. The peak current was slightly potentiated and "run-down" in the presence of the drug was 12%, 30 min later (n= 3), compared to 17% in control experiments. Currents are leak and capacity subtracted. Standard internal and external solutions were used. Cell 1506941.

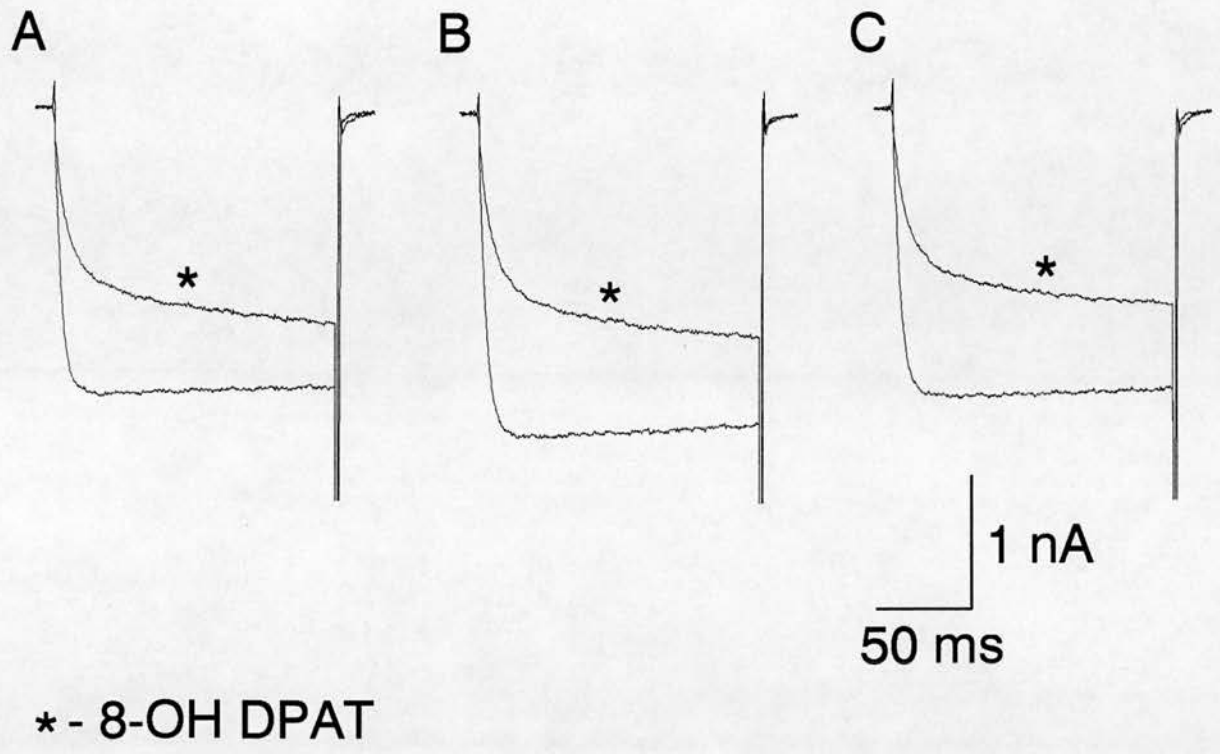

Figure 5.19: Protein kinases A and C are probably not involved in the modulation of calcium currents by 8-OH DPAT. Forskolin, a compound that stimulates intracellular cAMP that then probably activates cAMP-dependent protein kinase, slightly potentiated the peak Ca current compared to control DR neurones. 8-OH DPAT (50 μM, ✱) was still active and the peak current was reduced, on average, by 26% (A). With addition of OA to the neurones dialysed with forskolin, the peak current was further enhanced, and the inhibition by the $5\text{-}HT_{1A}$ agonist (✱) was unaltered (B). In (C) H-7, a more specific blocker of protein kinase C, failed to prevent the inhibition induced by 8-OH DPAT (✱). The peak current was reduced, on average, by 31%, as in control experiment. Cells 1406941, 1506943 and 0706943.

Chapter 6 Effect of protein phosphatase inhibition on calcium current kinetics

Effect of protein phosphatase inhibition on calcium current kinetics

With an introduction of protein phosphatase 1, 2A and 2B blockers intracellularly, Ca current kinetics had different properties than in control experiments. For that reason, okadaic acid, microcystin, calyculin-A and FK 506 have been tested and their effect on activation, inactivation and deactivation kinetics examined. The same methods were used, as for analysing the kinetics in control conditions, i.e. in the absence of these compounds. Namely, mono and double exponential functions were tested, together with Hodgkin and Huxley m^1 to m^4 models.

Effect of okadaic acid on activation kinetics

The HVA Ca current was activated with depolarising pulses using the standard voltage protocol, and the peak current amplitudes were elicited with voltage steps from V_H -100 mV to -10 mV, for 150 ms every 20s.

In Figure 6.1 (A) the activation of the peak Ca current at 20° C in the presence of OA is shown. In all DR neurones examined in the presence of OA, n= 45, it was clear that the best fit was a single exponential. The activation time constant, τ_a, was on average 2.85±0.09 ms. This is similar to the time constant obtained in control DR neurones, where, in the absence of OA, τ_a was 2.35 ms, and the best fit was also a single exponential.

In the same cell, the activation of the peak Ca current following a prepulse application is also shown. The prepulse evoked current that peaked to 105% of control current, i.e. in the absence of the prepulse. Time constant of the peak Ca current following a prepulse application was on average 2.57 ms. That is comparable

to the control cells, where τ_a, with an addition of a prepulse was 2.42 ms. In all cells tested, a monoexponential function provided the best fit. Unfortunately, the resolution of the instruments used in the experiments prevented the measurements of faster activation kinetics in a number of neurones and, therefore, the activation of the peak current in only 26% of cells (12 out of 45) was analysed.

Effect of temperature on activation kinetics

A temperature dependency was studied in the same cell, at 20° and 25°C, respectively, as shown in Figure 6.1 (B). As described above, in the DR neurones dialysed with OA, a single exponential was the best fit at 20°C. However, an increase in temperature for 5°C, dramatically affected the activation kinetics. Single exponential was still the best fit, but the activation was much faster. Regrettably, due to the problems with the settling time of the voltage-clamp in these studies, there were difficulties in analysing the activation kinetics of the peak current evoked at 25° and 30°C.

At 20°C, the best fit was obtained with m^2 Hodgkin and Huxley model, as in control experiments. The time constants for m^1 to m^4 models were 1.52, 1.31, 1.25 and 1.22 ms, respectively.

Effect of 5-HT_{1A} agonist on activation kinetics

It has already been shown, that an application of 8-OH DPAT (50 μM) into the external solution inhibited the peak Ca current in the presence, as well as in the absence, of OA. Therefore, it was to expect that the presence of OA would not change the activation kinetics recorded during the 8-OH DPAT application.

Figure 6.2 shows the effect before and during 8-OH DPAT (50 μM) application, with OA (1 μM) included in the recording pipette. In OA perfused cells, at 20° C, the time constant obtained was 2.35 ms and the best fit was monoexponential. However, in the presence of 8-OH DPAT, the best fit was not a single exponential any more, but, for 81% neurones tested, n= 17, that was a double exponential. In 17.6% of cells, 3 out of 17, the best fit was monoexponential. The time constant for a single exponential in the presence of 8-OH DPAT was on average 4.87±0.30 ms and that was almost 2-fold slower compared to control OA cells. For the double exponential fit, a fast time constant, $\tau_{a,f}$, was 3.22 ms, and a slow one, $\tau_{a,s}$, 20.6 ms. The best fit for Hodgkin and Huxley model was m^1, and not m^2, as in control OA cells.

Effect of prepulse on activation kinetics

In Figure 6.2, the effect of a prepulse on the activation kinetics is also shown. The action of the prepulse (3) on the peak Ca current activation was studied in the presence of OA and 8-OH DPAT. In all cells tested, the prepulse restored the activation kinetics to a single exponential fit. For Hodgkin and Huxley model, m^2 was again the best fit. The most significant observation was that the prepulse completely restored the activation time. The time constant, τ_a, following the prepulse application, was on average 3.35 ms, compared to 2.85 ms, in the cells dialysed with OA and in

the absence of 8-OH DPAT. That was significantly different than τ_a obtained in the presence of 8-OH DPAT, which was of 4.87 ms.

Effect of microcystin on activation kinetics

Effect of microcystin was tested using the same protocol as employed in OA cells. In the concentration of 1 μM, MC was included in the recording pipette and dialysed the cells. It has been described earlier, that, in DR neurones, MC strongly potentiated the peak Ca current and the fast "run-down" of the peak current was prevented.

Figure 6.3 shows that at 20°C, the best fit was a single exponential, as it was in OA cells. Time constant for the DR neurones dialysed with MC, was on average of 2.76±0.04 ms, n= 9, compared to τ_a of 2.85 ms obtained in OA cells. When Hodgkin and Huxley models were analyzed, m^2 was the best fit.

The effect of a standard prepulse application was then tested, and the activation time was slightly speeded up, with τ_a on average of 2.70 ms.

This similarity in the results obtained in the presence of okadaic acid and in the presence of microcystin was expected. Both of these compounds, belong to the same family of PP1 and 2A inhibitors. The action of MC was somehow stronger, and the reason might well lay in the fact that this compound is membrane impermeable and does not degrade as easily as okadaic acid does (Frace & Hartzell, 1993).

Activation kinetics of the peak Ca current in DR neurones in the presence of both MC and 8-OH DPAT was tested, as shown in Figure 6.4. In the presence of MC, τ_a was 2.76 ms. However, 8-OH DPAT increased the activation time and τ_a for a single

exponential was on average 5.41±0.04 ms, n= 6. That was a 2-fold increase, compared to control MC cells. A double exponential was the best fit in all 6 cells tested, together with m^1 of the Hodgkin and Huxley model.

In addition of a prepulse, the activation kinetics was restored, n= 4. The time constant was on average 2.83 ms and that was indeed very close to the time constant in control MC cell (τ_a of 2.76 ms). So, the effect of the prepulse seen in OA cells, was repeated in the presence of MC. Not unexpectedly, a single function was the best fit, in the presence of the prepulse.

Effect of GTP-γ-S on activation kinetics

It was important to study the action of GTP-γ-S, because its effect on the peak Ca current was similar to the 8-OH DPAT action. That 5-HT_{1A} ligand produced a double exponential activation curve and significantly increased the activation time. Both effects were examined in the presence of GTP-γ-S and OA, as shown in Figure 6.5. The activation time increased and the time constant, τ_a, was on average of 3.58±0.06 ms, n= 7 at 20° C (A). That result was similar as in the presence of 8-OH DPAT and OA, where the τ_a was of 4.87 ms. The best fit for all cells was a double exponential. In addition, m^1 was the best fit in 7/7 DR neurones.

A prepulse restored the activation kinetics and the best fit for 5/7 cells was a single exponential, as shown in the same Figure. The time constant in the presence of the prepulse was 3.21±0.04 ms.

To examine a temperature-dependency of the activation kinetics in the presence of GTP-γ-S and OA, the temperature was stepped from 20° C to 25° C, see Figure 6.5 (B). Unexpectedly, unlike in the cells dialysed with GTP-γ-S but without OA, the

activation was rapid and monoexponential, at 25°C (n= 3). In the presence of a prepulse, the activation curve was also best fitted with a single exponential, n= 3. This result suggests that OA speeded up the activation of the peak Ca current in DR neurones in the presence of GTP-γ-S. The effect of temperature was fully reversible. The temperature was returned to 20°C, and the current kinetics restored.

Calcium currents inactivation

In this section, the changes in inactivation kinetics are described, in cells dialysed with okadaic acid. It was observed that inactivation rate was very variable among DR neurones and even in the same neurone. In most DR cells, the current amplitude at the end of the test pulse (150 ms long) was not significantly different than the peak current. At 20°C, the current peaked on average at 2.53 nA, compared to 2.46 nA, at the end of the test pulse. Furthermore, inactivation was not recorded in all cells at 20°C, but with an increase in temperature to 25°C, inactivation was stronger and available for analysis. Maximal inactivation of the current was evoked at potentials around -10 mV. The same observation was reported by Frace and Hartzell (1993), in frog cardiac myocytes. Inactivation curves were fitted with mono and double exponentials.

As described earlier, the peak amplitude and activation kinetics of Ca current are strongly temperature dependent. With an increase in temperature, the current peaked at higher amplitudes and the activation time was shortened. As in control cells, in OA perfused neurones, stepping the temperature from 20° to 25°C speeded inactivation of the peak current. Figure 6.6 shows inactivation curves obtained in the cell dialysed with OA at 20° and 25°C, as indicated. It is clearly shown that at the higher

temperature, the inactivation was stronger. The difference in the rate of inactivation was best seen when the two time constants were compared. For 20° and 25°C, the time constants were 236 and 68 ms, respectively. That was really a dramatic effect where the increase in temperature of 5°C, produced an almost 3.5-fold faster rate of inactivation. The best fit at 20°C and 25°C was a single exponential in 27/29 and 4/4, respectively.

The result obtained contradicts the observation by Werz et al. (1993), who argued that OA increased the inactivation rate in dissociated bullfrog sympathetic neurones, compared to control cells. In control DR neurones, i.e. in the absence of OA, the time constants of inactivation at 20° and 25°C were 203 and 47 ms, respectively, which is, indeed, very close to the data obtained in the presence of OA. One difference might lay in the fact that in the sympathetic ganglion neurones, Ca current is 90% of the N-type, whereas only 40% in DR neurones. Furthermore, the group did not indicate the temperature at which their results were obtained and that could be a crucial difference, as shown here. They dissolved OA in DMSO, which could possibly by itself affect inactivation kinetics. As described earlier, DMSO potentiated the peak Ca current in DR neurones.

One other observation seen in OA cells was that a prepulse potentiated the peak Ca current and enhanced the activation rate. Therefore, it was curious to examine, what effect the prepulse had on inactivation of the current. However, at 20°C, the standard prepulse application produced just short of a significant change in the inactivation rate. Single exponential function was the best fit in all cells tested, n= 21. Time constant of the inactivation, τ_i, in the absence of the prepulse was on average 236 ms, whereas, with the prepulse, τ_i was on average 189 ms.

It was impossible to assess inactivation kinetics in control DR neurones dialysed with GTP-γ-S, since in most neurones the current was not fully activated at the end of the test pulse. In the presence of OA the current usually peaked during the test

pulse and inactivation kinetics were analysed.

Figure 6.7 shows the inactivation curves in the presence of GTP-γ-S and OA, at 20° C (A). The best fit was a double exponential. Time constant for a single exponential was of 449 ms. That was significantly different from τ_i value of 236 ms in control OA cells. With the addition of a prepulse, the inactivation kinetics was restored, and the best fit was a single exponential. That result is close to the one obtained in OA dialysed cells, with τ_i of 241 ms. Therefore, it appears, that a prepulse has not only speeded the activation, but also restored inactivation kinetics in the neurones dialysed with GTP-γ-S.

A very similar picture was obtained at 25° C (B), where both curves were monoexponential. Time constants, τ_is, were on average of 66 and 21 ms, respectively. It is, therefore, to conclude, that the inactivation rate was temperature-dependent and the application of the prepulse speeded up inactivation of the Ca current in DR neurones.

Calcium currents deactivation

As described previously, the amplitudes and kinetics of the tail current were extremely variable. With okadaic acid dialysing DR neurones, the speed of deactivation was even faster and caused substantial difficulties related to the data analysis. However, some data, obtained at 20° C, is shown here. As in the cells in the absence of OA, the best fit was a single exponential. The time constant of the tail current was, on average, of 2.23±0.09 ms, n= 16. That was faster than in control cells, where τ_t was of 2.77 ms ($p< 0.01$). Application of 50 μM of 8-OH DPAT in the presence of OA had no effect on the tail current kinetics. When the cells were dialysed with GTP-γ-S and OA two different effects were seen. In 3/7 cells, τ_t was

not different than in control OA cells, and measured on average 2.96 ms. On the other hand, in 4/7 cells, τ_t averaged 3.80 ms, that is significantly longer than in control OA neurones, $p< 0.001$. This variability illustrates the problem in analysing fast tail current kinetics in DR neurones dialysed with OA.

A summary of the effects on the peak Ca current and activation kinetics in control DR neurones, and in the neurones perfused with OA, microcystin, 8-OH DPAT and GTP-γ-S at different temperatures, is shown in Figures 6.8 - 6.11. It appears that 8-OH DPAT-induced down-modulation of the peak current is G-protein mediated. OA significantly enhanced the peak Ca current both in the presence and in the absence of 5-HT_{1A} receptor activation, but had no effect on the inhibitory action of 8-OH DPAT. In the presence of OA, a prepulse additionally increased the peak current, but did not completely reverse the reduction of the peak current induced by 8-OH DPAT or GTP-γ-S.

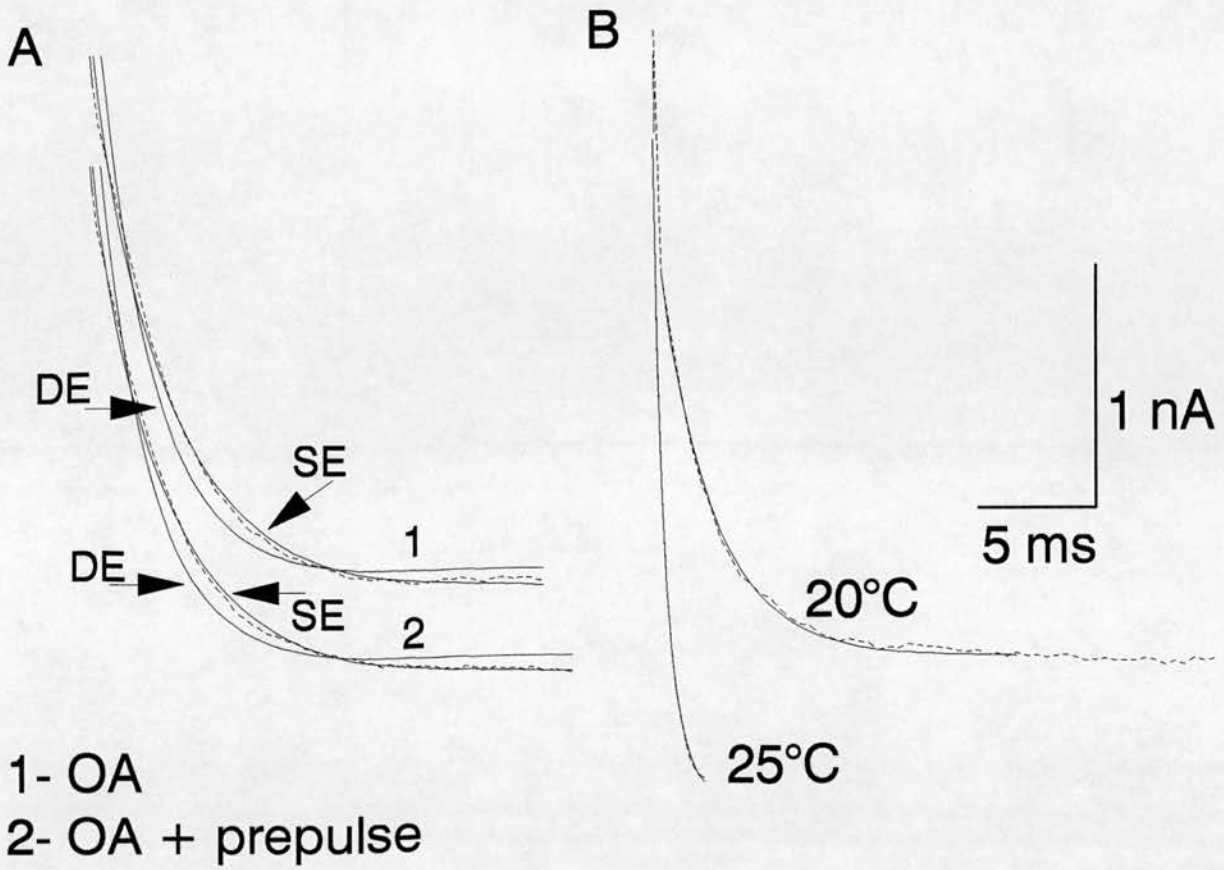

Figure 6.1: Activation of the peak calcium current in the presence of okadaic acid at 20° and 25°C. In (A) activation of the peak Ca current in the presence of OA (1) was best fit with a single exponential and τ_a was, on average, of 2.85 ms, n= 45. Following application of a prepulse (2) the activation time was slightly faster and τ_a was of 2.57 ms, n= 12. In (B) a step in temperature to 25°C, speeded up the activation of the peak current (n= 3). OA (1 μM) was included in the recording pipette and the peak current was evoked using standard voltage pulses. The activation of the peak current is shown as a broken line (···), and single (SE) and double (DE) exponentials are superimposed as single lines (—), as indicated. Cells 1105932 (A) and 1602941 (B).

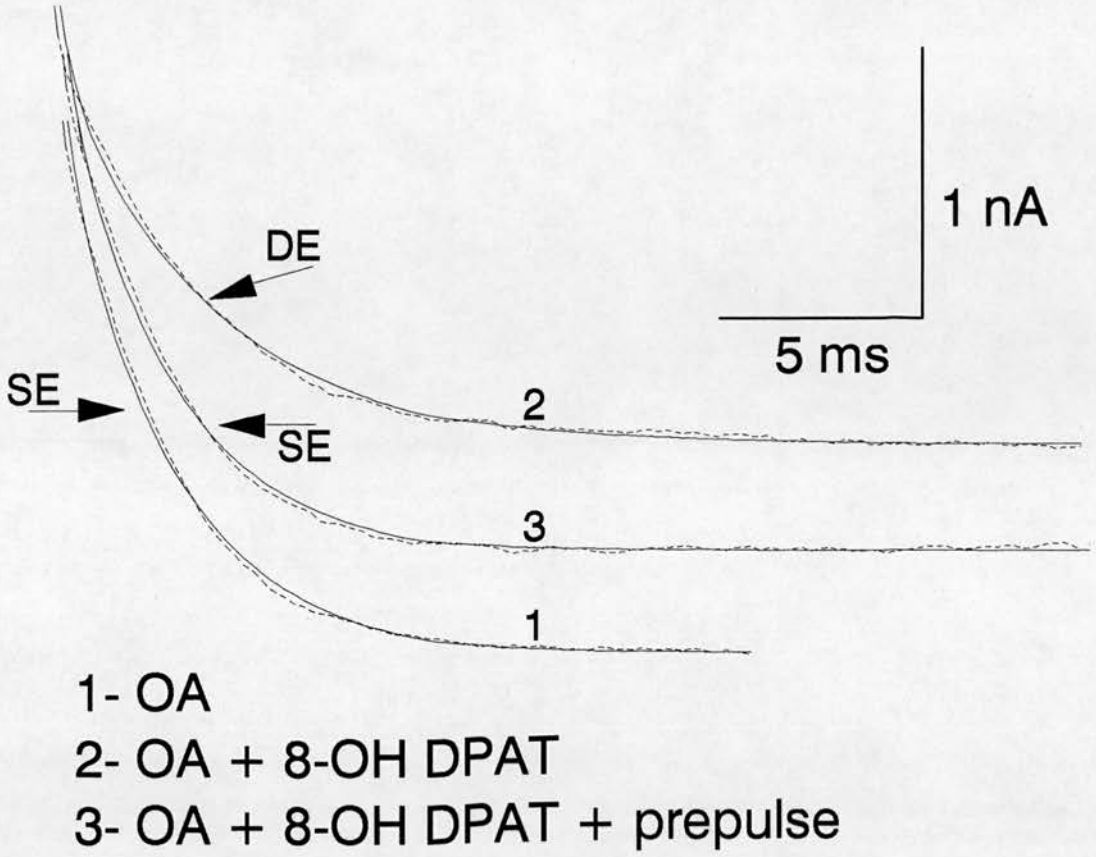

Figure 6.2: 8-OH DPAT prolonged the activation time of the peak calcium current in the presence of okadaic acid and the effect was restored by a prepulse. In DR neurones dialysed with OA (1) τ_a was of 2.85 ms and the best fit was a single exponential. 8-OH DPAT (50 μM, 2) was applied externally to the same neurone and the activation of the peak current was twice longer. The best fit was a double exponential with $\tau_{a,f}$ of 3.22 ms and $\tau_{a,s}$ of 20.6 ms, n= 14. However, application of a prepulse (3) in the presence of 8-OH DPAT, completely restored the activation kinetics. The best fit was a single exponential with τ_a of 3.35 ms. OA (1 μM) was added to the pipette solution and standard voltage steps were used. Both single (SE) and double (DE) exponential fits are shown superimposed on the peak Ca current. Cell 2402943.

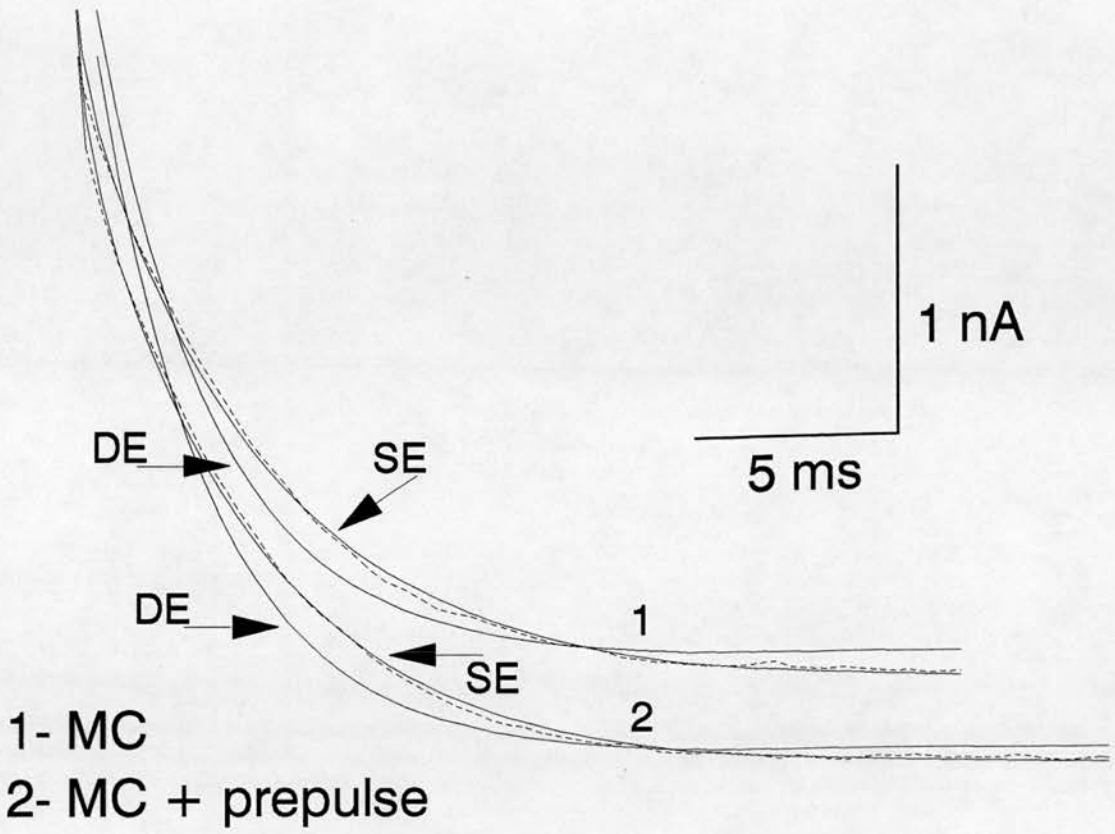

Figure 6.3: Activation of the peak calcium current in the presence of microcystin. Peak Ca current evoked in the presence of microcystin (1) was best fit by a single exponential with τ_a, on average, of 2.76 ms, n= 9. Application of a prepulse (2) also produced activation of the peak current that was best fit with a single exponential, and with similar τ_a of 2.70 ms. The effect is similar to that produced in OA dialysed neurones. MC (1 μM) was included in a standard internal solution and standard voltage pulses were used. Cell 1304932.

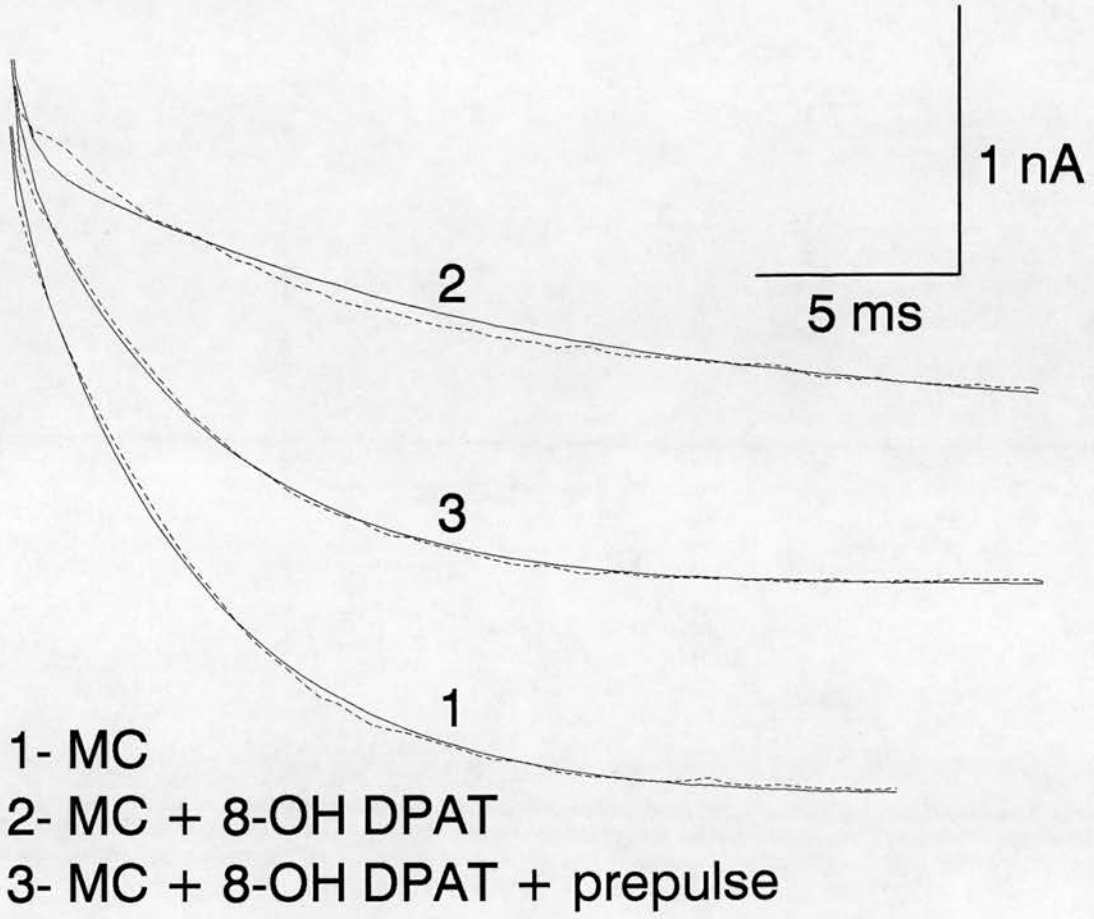

Figure 6.4: 8-OH DPAT induced slowing of the activation kinetics of the peak calcium current in the presence of microcystin, that is restored by a prepulse. At 20° C, the peak current activation in DR neurones dialysed with MC (1) was best fit with a single exponential and τ_a of 2.76 ms. However, 8-OH DPAT (2) significantly prolonged the activation time and the best fit was a double exponential, n= 6. Addition of a prepulse fully restored the kinetics to 2.83 ms, and a single exponential was the best fit. MC (1 μM) was included in the recording pipette and 8-OH DPAT (50 μM) was applied into the standard external solution. Cell 1304931.

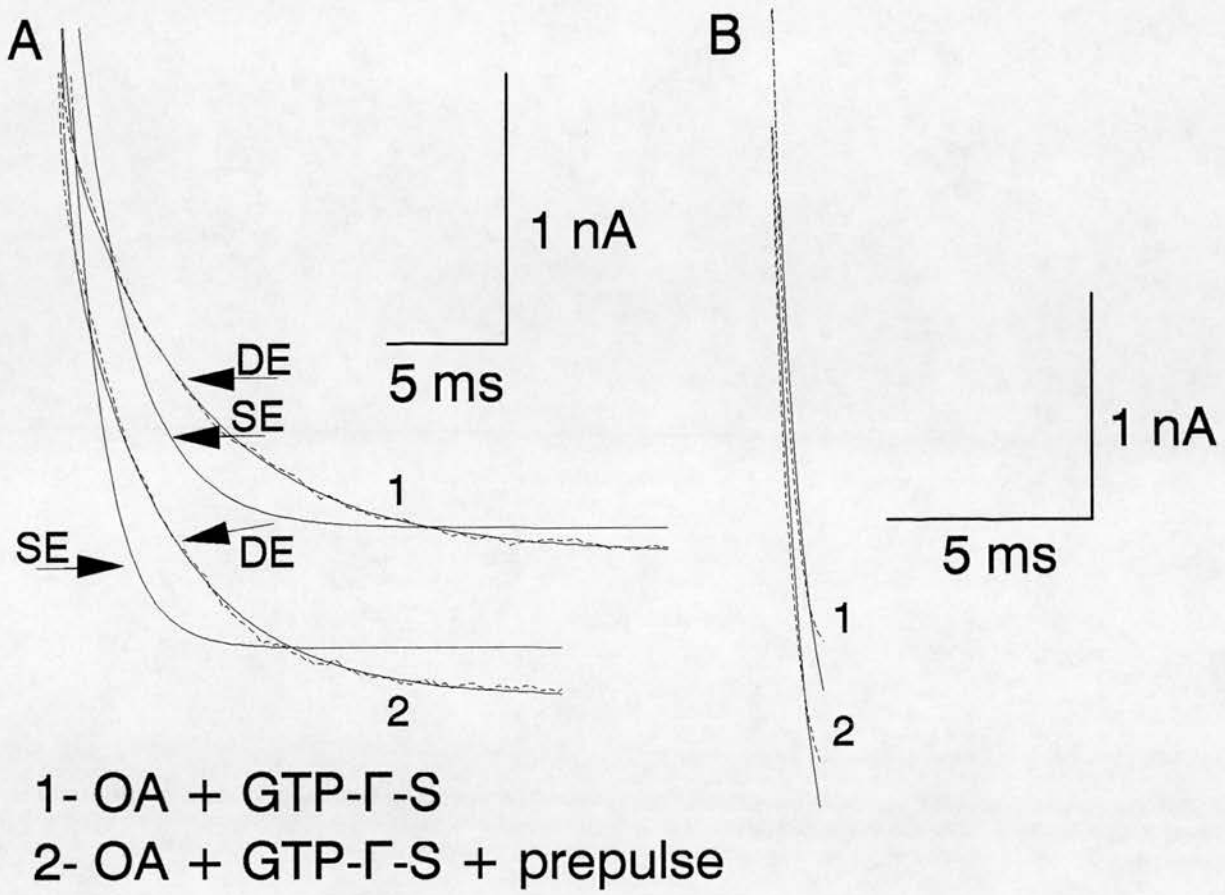

Figure 6.5: Effect of GTP-γ-S of the activation kinetics in the presence of okadaic acid at 20° and 25°C. GTP-γ-S significantly slowed the activation time compared to control OA cells and in that mimicked the effect of 8-OH DPAT. At 20°C (A), the best fit was a double exponential (n= 7, 1). With the addition of a prepulse (2), the kinetics was fully restored and the best fit was a single exponential with time constant of 3.21 ms, n= 5. In (B) the peak Ca current activation in the presence of OA and GTP-γ-S (1) and in addition of a prepulse (2) is shown at 25°C. The activation was monoexponential in both conditions, n= 3. GTP-γ-S (200 μM) and OA (1 μM) were included in the recording pipette. Both single (SE) and double (DE) exponentials are superimposed on the corresponding peak current traces. Standard voltage pulses were used. Note two different calibration bars for (A) and (B). Cell 1805941.

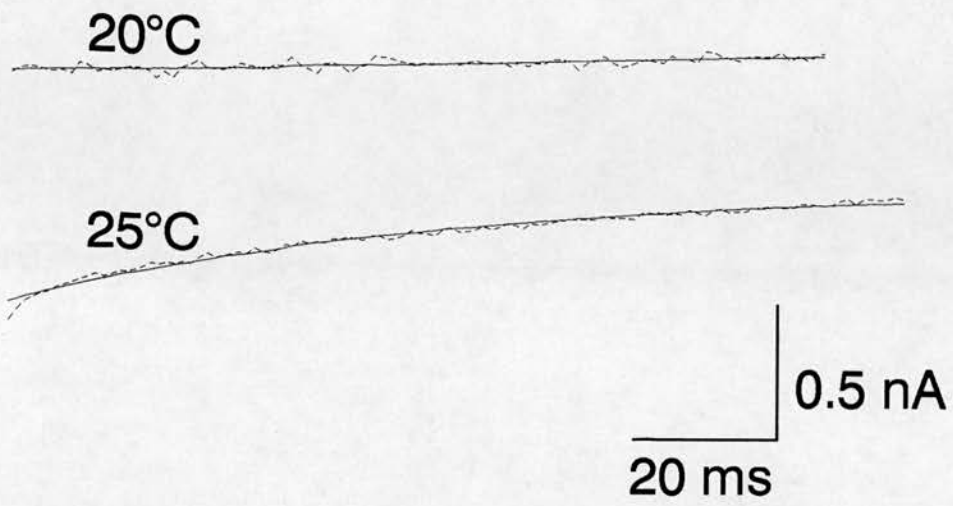

Figure 6.6: Inactivation of the peak calcium current in the presence of okadaic acid at 20° and 25°C. Increase in temperature from 20° to 25°C significantly speeded the inactivation of the peak current. The best fit at both temperatures was a single exponential and time constants obtained at 20° and 25°C were, on average, of 236 and 68 ms, respectively (n= 27 and 4, respectively). Standard internal and external solutions were used, and the peak currents were evoked by standard pulses. Cell 1606931.

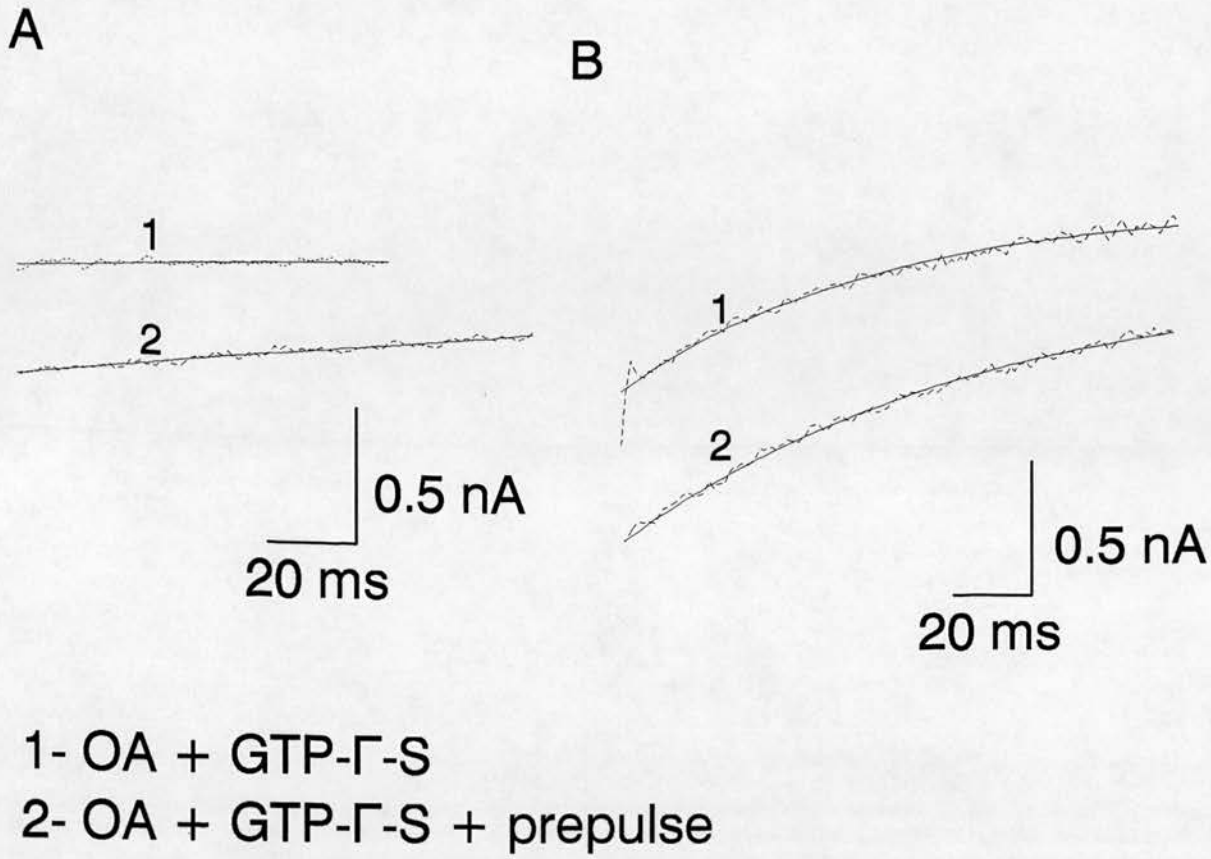

Figure 6.7: Inactivation of the peak calcium current in cells dialysed with okadaic acid and GTP-γ-S at 20° and 25°C. Peak Ca current in GTP-γ-S dialysed DR neurones inactivated only in the presence, but not in the absence of OA at 20°C. Moreover, inactivation was temperature-dependent, and the higher the temperature, the stronger was the inactivation. At 20°C (A) a double exponential (1) was the best fit and application of a prepulse (2) restored the inactivation kinetics to a single exponential, as in control OA cells. The best fit was a double exponential and for a single exponential obtained in the presence of the prepulse τ_i was of 241 ms. At 25°C (B) inactivation was faster aand nd monoexponential (1) with τ_i of 66 ms, and in addition of a prepulse (2) τ_i was of 21 ms. GTP-γ-S (200 μM) and OA (1 μM) were included in the standard internal solution. Standard external solution and voltage jumps were used. Cell 1805942.

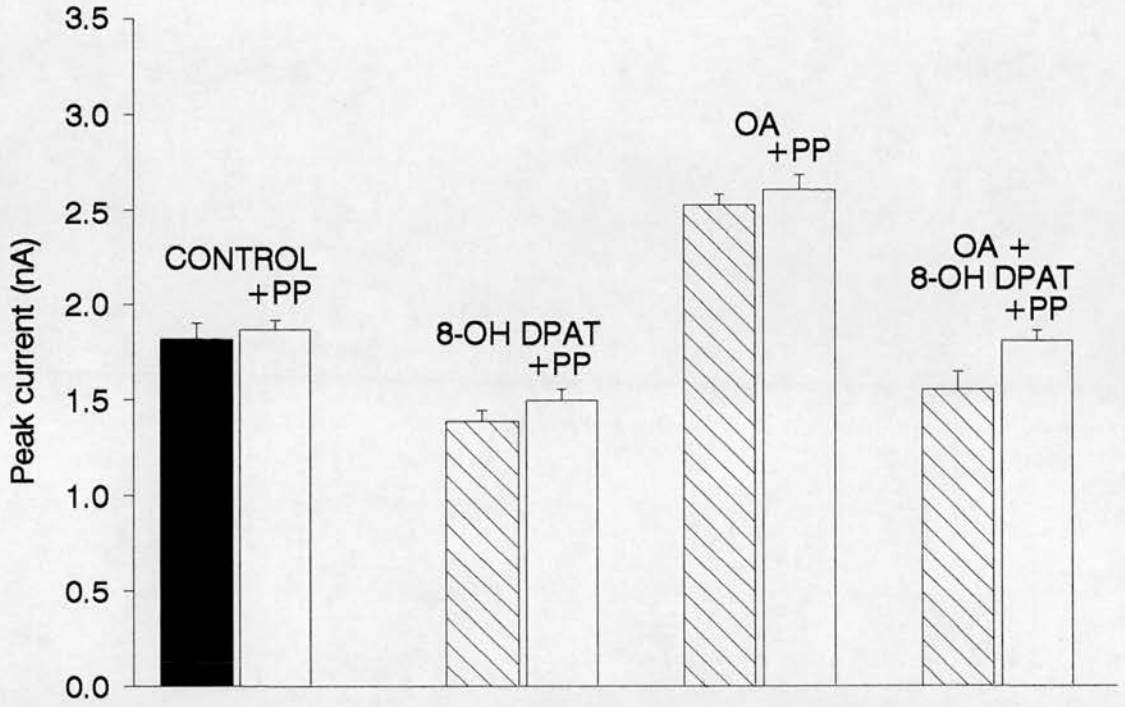

Figure 6.8: Summary of the peak calcium current amplitudes in control experiments and following the addition of 8-OH DPAT and okadaic acid at 20°C. Control Ca current (closed column) peaked at 1.82 nA in DR neurones. 8-OH DPAT (50 μM) decreased the peak current by 34% and this was partially reversed by a prepulse. OA (1 μM) significantly increased the peak Ca current by 39%, $p< 0.01$. In the presence of OA, the prepulse still potentiated the peak Ca current. The depression caused by 8-OH DPAT in OA dialysed neurones was only slightly less than in control cells, i.e. 31% of the peak current was inhibited. The open columns show the effect of the prepulse.

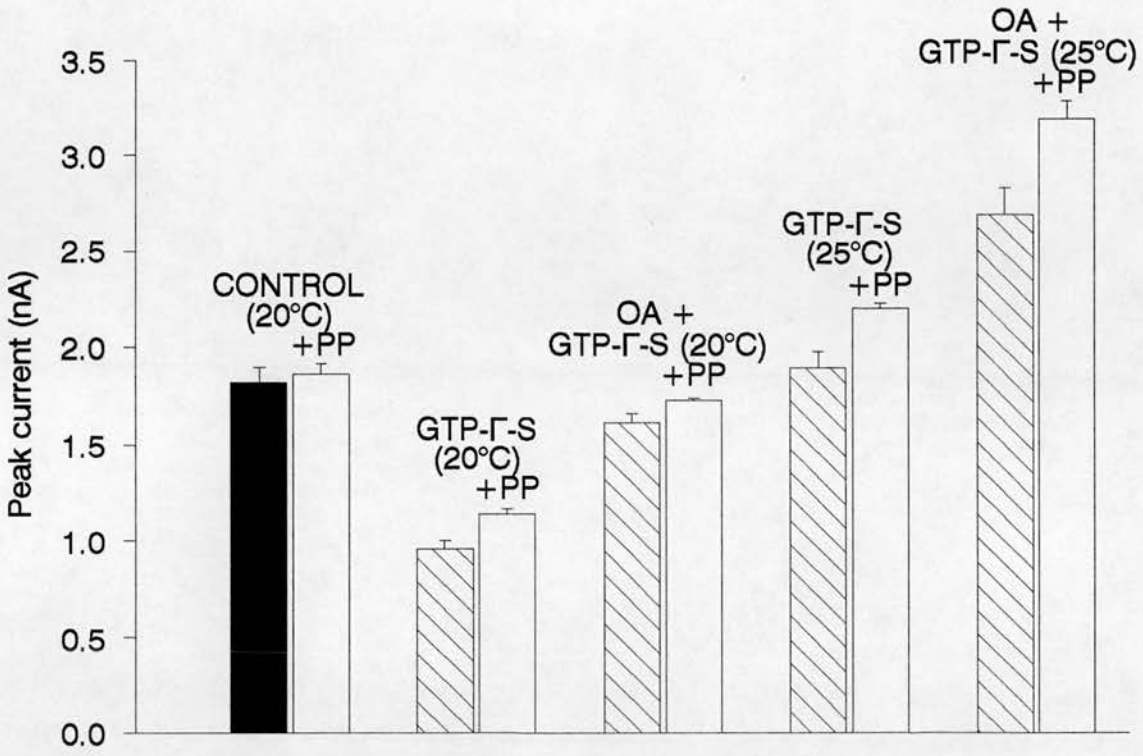

Figure 6.9: Summary of the peak calcium current amplitudes in control experiments and following the addition of GTP-γ-S and okadaic acid at 20° and 25°C. Control Ca current (closed column) peaked at 1.82 nA in DR neurones. Inclusion of GTP-γ-S (200 μM) in a pipette decreased the peak current by 53%. The effect was only partially reversed by a prepulse. At 25°C, the peak current was 1.90 nA, compared to 0.96 nA at 20°C. Addition of OA (1 μM) in the pipette, enhanced peak Ca current to 1.62 nA at 20°C, and 2.70 nA at 25°C, respectively. A prepulse (open columns) further potentiated the peak Ca current and the effect was more potent in the presence of OA.

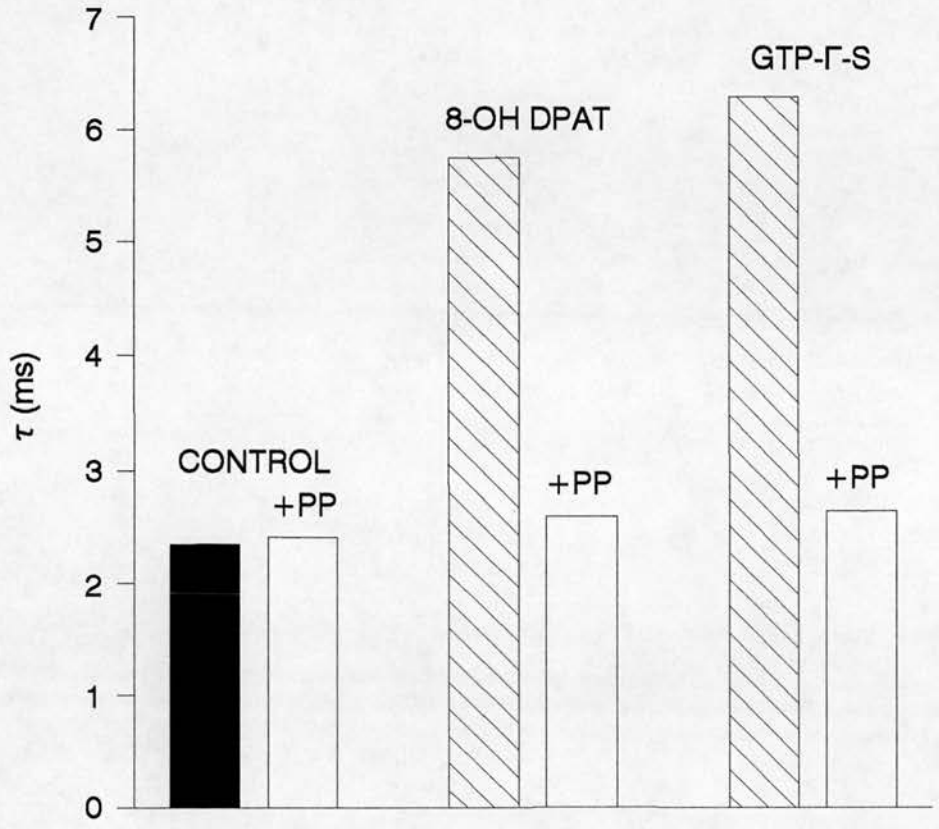

Figure 6.10: Comparison of the mean activation time constants, τ_a, for control, 8-OH DPAT and GTP-γ-S experiments, and the effect of prepulses. In control DR neurones τ_a was 2.35 ms (closed column). Bath application of 8-OH DPAT increased τ_a to 5.75 ms, and was similar in effect to that produced when DR neurones were dialysed with GTP-γ-S, 6.21 ms. In the presence of the prepulse (open columns), τ_a in control, 8-OH DPAT and GTP-γ-S perfused neurones was 2.42, 2.60 and 2.64 ms, respectively. Time constants used were best fit single exponentials.

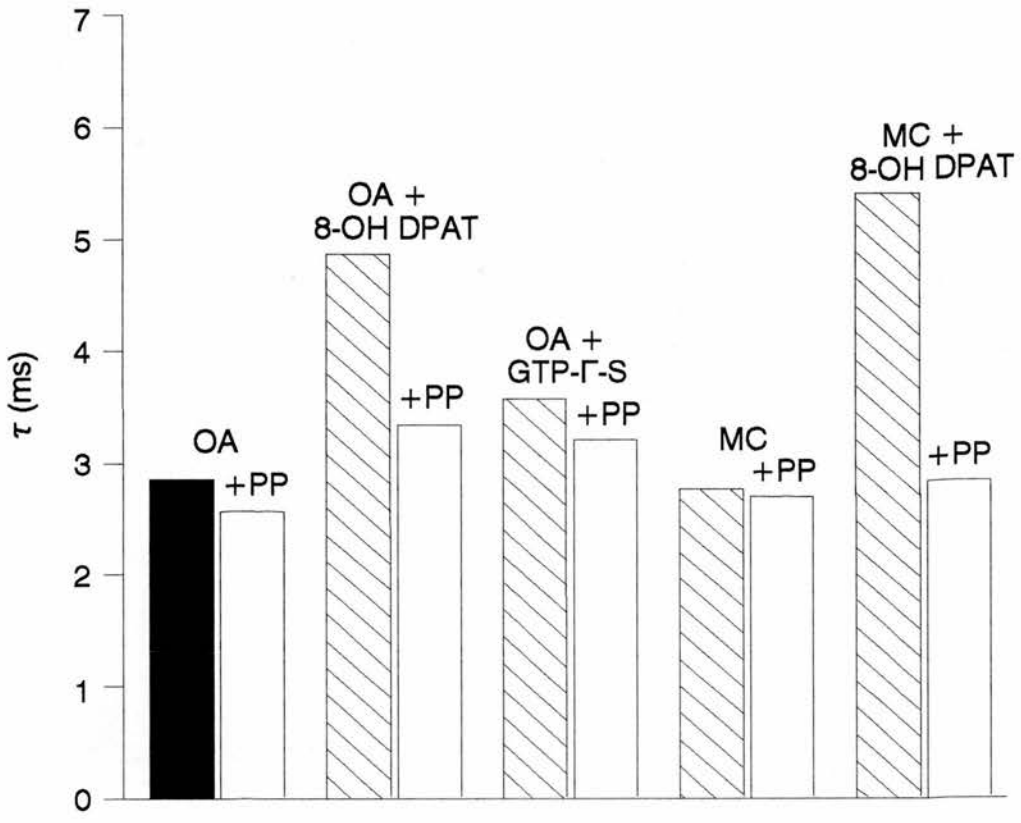

Figure 6.11: Summary of the rates of activation, τ_a, in DR neurones dialysed with okadaic acid and microcystin. The activation time constant for OA, MC and control cells was 2.85, 2.76 and 2.35 ms, respectively. Perfusion of the cells with 8-OH DPAT in the presence of either OA or MC increased the activation time to 4.87 and 5.41 ms, respectively. A prepulse produced transient restoration of the activation kinetics. In the neurones dialysed with OA and GTP-γ-S, Ca currents were not fully activated during a 150 ms long test pulses. Time constants used were best fit single exponentials. Drug applications are shown in closed or hatched columns, and the addition of a prepulse by open columns.

Chapter 7 Discussion

The major finding in this study is that micromolecular concentrations of the extracellularly applied 5-HT_{1A} agonist 8-OH DPAT down-modulate HVA Ca channels in dorsal raphé neurones, and suppress their gating kinetics. These effects appear to be G-protein modulated. Furthermore, the HVA Ca current is up-regulated by inhibition of dephosphorylation, and this modulation is enhanced in the presence of conditioning depolarising pulses. However, the present data suggest that 8-OH DPAT and phosphatase inhibitors use two different, but probably closely related mechanisms for their action on the HVA current.

Properties of calcium currents in dorsal raphé neurones

High-voltage activated Ca current, evoked as the peak current was investigated in this work. With an advantage of using a whole-cell voltage-clamp technique, intracellular and extracellular solutions were designed to isolate Ca currents and completely block outward sodium and potassium currents. In addition to that, high concentration of EGTA, as an internal Ca^{2+} chelator, and Ba^{2+} as the charge carrier were employed. Ba^{2+}, unlike Ca^{2+}, does not activate Ca-dependent intracellular processes and it is more stable when tested at different temperatures. Moreover, it has been suggested that N- and L-type Ca channels that are present in DR neurones are slightly more permeable to barium, than to calcium ions (Hess, 1990).

Although the peak current was abolished in the presence of Cd^{2+}, a specific Ca channel blocker, relatively high doses of the drug were required. Fox et al. (1987) used 20 μM of Cd^{2+} to fully suppress Ca current in DRG cells. Allen et al. (1993) reported that Cd^{2+} in lower concentrations (< 1 μM) markedly inhibited HVA, and in higher concentrations (1-200 μM) both HVA and LVA currents in rat forebrain

neurones. In DR neurones the concentration of Cd^{2+} needed to completely abolish the current was about 50 times higher than used by others (Fox et al. 1987b; Gross & Macdonald, 1987; Yang et al. 1993), but similar to some (Boland & Dingledine, 1990). One possible explanation can be that perfusion of the drug was too slow and short (cells were exposed to Cd^{2+} for a maximum of 10 min) or that the compound some how "missed" the tested cell. However, this is unlikely, because other compounds were applied in the same fashion. For example, 8-OH DPAT inhibited the peak Ca current in 92.3% of neurones studied, which corresponds to the number of DR neurones that possess 5-HT_{1A} receptors (~90%). Thus, a flow artifact seems not to present a major problem in these experiments. Another possibility is a differential effect of Cd^{2+} on HVA and LVA Ca currents. Penington and Kelly (1990) have shown at least four types of Ca currents to exist in DR neurones. Most of the characterised current is N-type (~40%), and a smaller proportion is L-type (~0-20%) and T-type current. Moreover, at V_H -50 mV, where all T-type current is inactivated, an application of ω-CgTx and the DHP antagonist nimodipine into the bath solution, blocked only 50% of the HVA current. Thus, ~45% of the Ca current remains unblocked (Penington et al. 1991). As described above, there is evidence to suggest that LVA current is less sensitive to Cd^{2+} (Allen et al. 1993). This might indeed be at least partial explanation for the result obtained. The small part of Ca current that was relatively insensitive to higher concentration of Cd^{2+} is probably LVA current, although this was not specifically tested in the presence of Ni^{2+} or amiloride. Furthermore, it is also likely that other, still unidentified HVA Ca current type shows lower sensitivity for Cd^{2+}.

Voltage-activated Ca current showed a temperature-dependency in DR neurones. With a temperature step of 5°C, as from 20° to 25°C, the current peaked significantly higher, by 68%, and the activation kinetics was speeded up. This is similar to the observations by McAllister-Williams (1992) in the same neurones. The temperature-dependency was the strongest at the potentials that evoked the peak Ca

current. At higher temperatures, a shift in the current-voltage relationship to the hyperpolarising potentials was shown. For example, at 25° C, the peak current was elicited with a step to around -20 mV, compared to the potential of -10 mV at 20° C. An increase in temperature appears to shift the equilibrium of Ca channels toward opening. This leftward shift in the voltage-dependency of Ca currents may involve changes in a phosphorylation state of the channels, as a possible mechanism of modulating the Ca currents. A similar shift in the current-voltage relationship has been reported in other cell types (Nobile et al. 1990; VanLunteren et al. 1993).

Modulation of calcium currents by 5-HT_{1A} receptor activation

Calcium channels have an important role in neurotransmission in central and peripheral nervous system and they are found in all excitable cells so far studied. Given the ubiquitous nature of Ca channels, it is possible that they have an essential role in neuronal plasticity. In DR neurones, application of 8-OH DPAT led to a significant, but incomplete suppression of the peak Ca current by 34%, and the inhibition was relieved following a prepulse, by 60%. It is interesting that the reduction of the peak Ca current amplitude by 8-OH DPAT was slightly less than the depression of the current following 5-HT application (57%), that was observed in the same cells (Penington et al. 1991). One possible explanation for lower potency of 8-OH DPAT, compared to the effect of 5-HT is that while 5-HT is a full agonist on the 5-HT_{1A} receptors, 8-OH DPAT, arguably, acts only as a partial agonist.

In many neuronal types N-type channel was the proposed site of action for neurotransmitters because the transient component of Ca current was lost and that component was DHP insensitive. For example, Koike et al. (1994) showed that 5-HT and 8-OH DPAT blocked specifically N-type Ca current in hypothalamic neurones.

They proposed that the N-type channel regulates release of 5-HT, by acting on 5-HT_{1A} receptors. Other groups have also argued that the slowing of the activation kinetics observed in the presence of neurotransmitters results from a selective inhibition of a rapidly inactivating N-type current, leaving a slowly activating L-type current untouched (Wanke et al. 1987). However, in a number of different neuronal types N-type current does not always activate rapidly, nor does L-type current activate slowly (Jones & Elmslie, 1992). Moreover, in DR neurones the amount of transmitter-resistant current is considerably larger than the amount of L-type current, following application of 8-OH DPAT. Furthermore, Ciranna et al. (1993) showed that 8-OH DPAT inhibited Ca current in porcine melanotrophs. In these cells the drug selectively inhibited L-type current. In other cells, such as in snail *Helix aspersa* neurones, 5-HT potentiated Ca current (Paupardin-Tritsch et al. 1986; Hill-Venning & Cottrell, 1992) and specifically acted on T-type current (Berger & Takahashi, 1990).

It was shown earlier that in DR neurones the current inhibited following 8-OH DPAT application is only partially of N-type. 8-OH DPAT-induced down-modulation of the peak Ca current is reduced by only 10% in the presence of the N-type channel blocker ω-CgTx (Penington et al. 1991). Therefore, a significant amount of the peak Ca current that is blocked by 8-OH DPAT might be ω-CgTx and DHP insensitive and has to be characterised. This unidentified HVA Ca current is in many ways similar to N-type current in DR neurones. Some of the properties of this current are that it is a high-voltage activated current, it is transient with faster inactivation rate, the current is ω-CgTx and DHP insensitive and needs negative holding potential to reprime completely (Penington et al. 1991). Therefore, the inhibited current was either N-type with fast inactivation time constant and less sensitive to ω-CgTx, but sensitive to 5-HT, or the channel was sensitive to 5-HT, but it was not N-type channel. The latter hypothesis suggests that depression of the peak Ca current caused by 8-OH DPAT may affect more than one type of Ca channels. Furthermore, this observation shows that the pharmacological properties of DR neurones are quite

complex.

Interesting observation is that a whole host of neurotransmitters modulate Ca current interacting with G-proteins. In DR neurones, intracellularly applied GTP-γ-S mimicked the effect of 8-OH DPAT, both by down-modulating the peak current amplitude and slowing of the activation kinetics.

Furthermore, 8-OH DPAT applied to the DR neurones dialysed with GTP-γ-S produced no additional effect on the peak current amplitude or activation time. A similar modulatory pathway has already been examined in other cell types, such as rat pituitary GH_3 cells (Kleuss et al. 1991). Exogenously added G-proteins can restore the ability of neurotransmitters to inhibit Ca currents. Such experiments have generally suggested that G_o is more effective than G_i in the restoration experiments. It is known that there is an abundancy of G_o proteins in both central and peripheral nervous system, and this subtype of G-proteins is limited to neuronal, neuroendocrine and endocrine cells (Hescheler et al. 1987; Ewald et al. 1988b). For example, in DRG neurones Campbell et al. (1993) showed that G_o, but not G_i subtype was responsible for the modulation of Ca channels by baclofen. In a rat pituitary tumour cell line that possesses no G_o, dopamine had no effect on prolactin release, whereas in another cell line containing G_o, dopamine was effective (Dolphin, 1990).

Another example that G-proteins are required for calcium currents modulation was observed following a dialysis of DR neurones by GTP. In the absence of GTP, the peak Ca current decreased more quickly during recordings. Furthermore, the compound was needed for Ca channels to maintain sensitivity to modulation by 8-OH DPAT. A similar observation was made in other cell types (Elmslie et al. 1993).

Effect of prepulse on calcium currents amplitude and activation kinetics

A strong, conditioning depolarisation applied just prior to the test pulse had no effect on the peak current amplitude (2% increase in the peak current) nor on the activation kinetics (both were best fitted by a single exponential) in control DR neurones. In the presence of 5-HT_{1A} receptor activation a prepulse only partially relieved the inhibition of the peak Ca current in majority of the neurones. The block of the peak Ca current was never 100% relieved, and that was similar as observed in other cell types (Dolphin et al. 1989; Boland & Bean, 1993).

However, a prepulse had a significant effect in restoration of the activation kinetics. Application of the 5-HT_{1A} agonist 8-OH DPAT or an intracellular dialysis of DR neurones with GTP-γ-S produced a slowing of activation of the peak current. When the activation kinetics was analysed in DR neurones, a single exponential, which was the best fit in control neurones, was replaced by a double exponential in the presence of either 8-OH DPAT or GTP-γ-S. Following application of a prepulse to 40 mV, the activation kinetics was fully restored. A single exponential was the best fitted, with similar time constant as in control cells.

It is possible that these two effects observed in the presence of 5-HT_{1A} receptor activation, that is a reduction of the current amplitude and slowing of the activation kinetics, are two separate processes with similar time courses. Use of such two-pulse protocol as in the present study, i.e a prepulse preceding the test pulse, clearly illustrates that the 8-OH DPAT-induced slowing of the current activation can be completely reversed by conditioning depolarisations. The results also demonstrate, however, that the reversal of the current amplitude at depolarised potentials is generally incomplete, leaving the inhibition present even at strongly depolarised voltages. Therefore, it is possible to distinguish two components that presumably use distinct pathways. The slowing of the activation of the current is fully reversed at depolarised voltages, and this component is voltage-dependent. It appears that this component subsides with time, and its contribution to the total inhibition declines

during a depolarising pulse, see Figures 3.12, 3.17 and 3.18. The second component, that presents a reduction of the current amplitude, is not associated with a change in activation kinetics and is present throughout the duration of a test pulse. It is not reversed at depolarised voltages, i.e. it is voltage-independent. Although the two components can be separated on the basis of their voltage-dependency, they appear to be indistinguishable in their time course for onset and recovery. In support of this hypothesis it has been shown in a few cell types that a neurotransmitter inhibited Ca current without changing activation kinetics. This was shown in both central (Toselli et al. 1989; Sah, 1990) and peripheral (Forscher et al. 1986; Ewald et al. 1988b; Bean, 1989a) neurones. The voltage-dependence of transmitter action is confined to currents that demonstrate measurable slowing of the activation kinetics. By contrast, currents in which the kinetic slowing could not be measured demonstrate little voltage-dependency of neurotransmitter action (Aosaki & Kasai, 1989; Kasai, 1992). Furthermore, some G-proteins that mediate inhibition of Ca current have also been shown not to be associated with a slowing of the activation kinetics (Ewald et al. 1988b; Sah, 1990; Beech et al. 1992). Thus, it is possible that different G-proteins mediate slowing of the activation kinetics and inhibition of the current amplitude. It has already been shown that a single neurotransmitter can activate more than one class of G-proteins. For example, following application of muscarine PTX sensitive and PTX insensitive G-proteins are activated (Nathanson, 1987; Ashkenazi et al. 1989). Moreover, a PTX sensitive G-protein can contain different subtypes that can specifically be activated. Ewald et al. (1989) observed differential effects of PTX sensitive G-protein α-subunit subtypes, such as α_o, α_{i1} and α_{i2}, following application of neuropeptide Y and bradykinin in rat sensory cells. Diversé-Pierluissi et al. (1995) suggested that NE-induced inhibition of voltage-independent steady-state component of Ca current is mediated via G_i, whereas slowing of the activation kinetics follows activation of G_o, in embryonic chick DRG neurones.

The mechanism of the prepulse action is not fully understood. One hypothesis

might be that following a strong depolarisation, an "inhibitory molecule" unbinds from the channel in a voltage-dependent fashion (Golard & Siegelbaum, 1993). In control cells, i.e. in the absence of neurotransmitters, a majority of Ca channels reside in a closed state from which they are "willing" to open upon depolarisation. Possibly, addition of neurotransmitters increases the fraction of channels in the "reluctant" mode, thus resulting in a biphasic activation curve (Bean, 1989b). A slow activation in the presence of a transmitter shows the slow conversion of "reluctant" to "willing" channels. It is possible that in the presence of saturating doses of neurotransmitters a fraction of the Ca channels, which are shifted to the "reluctant" mode requires stronger depolarising pulses to open. According to this hypothesis, large depolarisations cause a number of "reluctant" channels to revert transiently to the "willing" mode.

In addition, others suggested that transmitters could modulate calcium currents by different intracellular mechanism. For instance, this process could depend on the buffering system used, suggesting a role of internal calcium in mediating these events. When intracellular BAPTA is low (0.1 mM) a neurotransmitter causes inhibition of the current amplitude and slowing of the activation of the current, whereas only slowing of the activation kinetics is present in 10 mM BAPTA (Bernheim et al. 1991; Beech et al. 1992). In rat hypothalamic neurones 20 mM of BAPTA completely prevented the inhibition of the current and slowing of the activation kinetics following application of L-glutamate (Zeilhofer et al. 1993). Qualitatively similar results were obtained with either Ca^{2+} or Ba^{2+} as the charge carriers.

Thus, it is possible to argue that the suppression of Ca current amplitude following 5-HT_{1A} receptor activation is probably due to a voltage-independent switch of the "willing" Ca channels to "reluctant" mode, whereas a voltage-dependent shift is responsible for the observed changes in the activation kinetics.

Inactivation of calcium currents

High-voltage activated calcium current in DR neurones inactivated slowly and only partially during voltage steps lasting 150 ms. Inactivation was the strongest at voltages that evoked the highest Ca currents. Inactivation, as well as activation, of the peak Ca current in DR neurones was temperature-dependent. At 20°C not all cells exhibited inactivation and the current at the end of the test pulse was insignificantly smaller than the peak current (1.75 nA compared to 1.82 nA, respectively). With an increase in temperature for 5°C, as from 20° to 25°C, the inactivation was dramatically faster, up to five times, $p< 0.001$. However, a great variety of inactivation rates was observed at both 20° and 25°C. It was described earlier (Penington et al. 1991) that ω-CgTx sensitive N-type channels, that form a majority of Ca channels in DR neurones, have excessively variable inactivation rates and might greatly influence inactivation in the neurones, in addition with other Ca channel types. Thus, N-type channels may be able to switch to various inactivation states and it is possible that stronger inactivation of the peak Ca current observed at 25°C may be due to the activation of channels favouring a fast inactivation state at higher temperature. In addition, it seems plausible that variable inactivation rates observed in DR cells are a measure of the mixture of multiple Ca channels types involved. In support of this view is the observation that inactivation is voltage-dependent.

Other groups (Brown et al. 1981; Kay, 1991) observed slower inactivation of Ca current in CA1 hippocampal neurones and *Helix aspersa* neurones, than in DR neurones. This contradictory finding might result from using Ca^{2+} as the charge carrier and strong buffering of internal calcium ions by BAPTA in their experiments. In the present experiments, the weaker Ca^{2+} chelator EGTA was used and Ba^{2+} as the charge carrier. Inactivation can also depend on different phosphorylation states of calcium channels in these cell types. There are indications to suggest that phosphorylation enhances voltage-dependent activation of Ca channels, and that

inactivation is due to their dephosphorylation (Chad & Eckert, 1986).

In DR neurones, inclusion of OA in the recording pipette produced just short of a significant change of inactivation. Time constant of inactivation in control DR neurones was 203 ms, compared to 236 ms in the presence of the phosphatase inhibitor. In sympathetic ganglion neurones, inactivation was stronger in the cells perfused with OA (Werz et al. 1993). As described, by evoking high-voltage Ca current, mostly N-type current inactivates. Thus, this difference between inactivation rates may be in the fact that in the sympathetic ganglion neurones Ca current is 90% of the N-type, whereas 40% in DR neurones. Moreover, Werz et al. (1993) does not indicate the temperature at which they obtained the result, and they dissolved OA in DMSO, which could possibly by itself modify the Ca current, see Figure 2.5.

"Run-down" of calcium currents

During whole-cell recordings a slow and irreversible decrease of Ca current amplitude, i.e. "run-down", occurred. This presents one of the disadvantages of the whole-cell technique.

It appears that "run-down" is a complex process that depends on at least two events: 1. loss of the intracellular source of energy, and 2. increase in intracellular Ca. In DR neurones, the rate of "run-down" was slower in the presence of ATP, showing that a progressive loss of high energy compounds from the cytoplasm is involved in the decay of the Ca current. Thus, this effect of ATP suggests phosphorylation to be an important factor in maintaining Ca currents (Elmslie et al. 1993; Tiaho et al. 1993). Indeed, when DR neurones were dialysed with phosphatase blockers, "run-down" was significantly smaller. For example, in the presence of OA "run-down" was 8% 30 min later, compared to 17% in control DR neurones, $p < 0.01$.

A similar phenomenon was observed in other cell types (Hescheler et al. 1988; Reinhart et al. 1991; Werz et al. 1993), but not in all (Kameyama et al. 1986).

An increase in internal Ca concentration could also cause a decline of Ca current. Addition of Ca chelator EGTA to the recording pipette significantly prolonged the currents survival, whereas the cell superfusion with caffeine (which releases Ca from sarcoplasmic reticulum and other internal stores) accelerated decay of the current (Belles et al. 1988). Byerly and Moody (1984) showed that the presence of both ATP and EGTA prevents fast "run-down" of Ca current more than the presence of high dose of EGTA alone.

Phosphorylation of the cells has been shown to protect some intracellular proteins against enzymatic hydrolysis (Belles et al. 1988). During longer recordings of Ca current and subsequent elevation in internal Ca concentration, Ca-dependent proteases could activate and cause proteolytic degradation of Ca channels. For instance, Chad and Eckert (1984) have shown that leupeptin, an inhibitor of Ca-dependent proteases prevented fast "run-down" of Ca current, however, only in the presence of phosphorylating agents. This involvement of enzymatic mechanism in the maintenance of Ca currents might also explain some of the temperature sensitive features of the Ca currents in DR cells. By increasing the temperature from 20° to 25°C "run-down" was significantly faster, whereas at 15°C the current was more sustained.

Modulation of calcium currents by protein phosphatase inhibition

Multiple phosphorylation sites on Ca channels are known to exist (Takahashi et al. 1987). However, the effect of phosphorylation on Ca channel activity is not fully understood. In DR neurones, protein phosphatase inhibition prevented the fast "run-

down" of the peak Ca current, potentiated the peak current and the effect was additionally enhanced in the presence of conditioning depolarisations. These findings suggest that phosphorylation stabilised the Ca current. In the presence of phosphatase 1 and 2A blockers a significant potentiation of the peak current occurred. Calcineurin, on the other hand, is dependent on calcium ions intracellularly, that were well buffered with EGTA, and it is unlikely that this phosphatase plays a major role in regulation of Ca current in DR cells. Thus, it appears that Ca channels were already phosphorylated to a great extent, however, additional phosphorylation was still possible. Therefore, it is possible that at any particular time Ca current amplitude is the product of dynamic equilibrium of phosphorylation and dephosphorylation cycle. It is presumed that in DR cells dephosphorylation is maintained by a basally active phosphatase and low protein kinase (PK) activity. Upon activation of PK, the equilibrium shifts toward a phosphorylated state due to deactivation of phosphatase activity.

Furthermore, in the presence of the phosphatase blockers, the sensitivity of the peak current amplitude to voltage was shifted to the left (more hyperpolarising level), suggesting a shift in voltage as a possible mechanism of enhancement of the peak current. It has been suggested in other cell types that Ca channels can be regulated by a voltage-dependent phosphorylation (Artalejo et al. 1992a). In chromaffin cells, strong depolarising pulses potentiated Ca current, possibly either by activating a protein kinase or by placing Ca channels into a conformation which is a better substrate for a kinase. It is not clear whether phosphorylation modulates Ca channels directly, i.e. as a result of phosphorylating its α- and/or β-subunits. There is a possibility that phosphorylation of the β-subunit facilitates the coupling between movement of gating charge and opening of the channel pore (Bean, 1994a). In cardiac myocytes agents that increase cAMP cause phosphorylation primarily of the β-subunit (Haase et al. 1993).

It appears that the role of phosphorylation is much clearer in heart muscle cells than in neuronal tissue. Hescheler et al. (1988) observed that OA produced

stimulatory effect on Ca currents in cardiomyocytes by suppressing dephosphorylation, and the process resulted in enhanced phosphorylation of channel related proteins, which in turn increased the opening probability of the channels. Increased opening probability and decreased channel closing rates induced by phosphorylation of Ca channels have also been shown in cell-attached patches in single-channel recordings from isolated rabbit ventricular myocytes, with Ba^{2+} as charge carrier (Ono & Fozzard, 1993).

It is shown that in DR neurones the inhibition of protein phosphatases 1, 2A and 2B has no effect on 8-OH DPAT-induced down-regulation of the peak current. The current was still enhanced, in addition to the inhibition by 8-OH DPAT (31%), similar to that in control cells (34%). Thus, it appears that the inhibitory effect of neurotransmitters on Ca current is not mediated via phosphorylation mechanism. The inhibition of the protein phosphatases does not prevent the effects of 8-OH DPAT and GTP-γ-S on Ca current. Similar observation was made in other neuronal cell types (Frace & Hartzell, 1993; Werz et al. 1993). For instance, Elmslie et al. (1993) showed that norepinephrine inhibited Ca current in the presence of OA. This group also showed that AMP-PNP applied instead of ATP preserved the NE response, thus suggesting that NE does not act by inducing phosphorylation of Ca channels. This compound has high affinity for many ATP binding sites, but it is not a substrate for protein kinases. They suggested that an ATP binding site needs to be occupied for successful coupling of the adrenergic receptor and Ca channel. The possibility for its localization include the receptor, G-protein and Ca channel, however, none of them has been shown to bind ATP. It is possible that a novel ATP-binding protein is involved in receptor-channel coupling.

Role of second messengers

As described, it appears that the phosphatase inhibitors modulate Ca current by inhibiting dephosphorylation and not via a different mechanism that do not require phosphorylation, because their effect is lost in the absence of ATP. Furthermore, it has not been shown that they can directly activate protein kinases. A proposed mechanism of OA action is that the drug inhibits protein phosphatase 1 and 2A and that presumably leads to an activation of serine/threonine group of protein kinases (Sassa et al. 1989). However, the exact protein kinase(s) that mediate this process are yet to be identified. Hunter (1987) suggested that mammalian cells are able to express over fifty different serine/threonine kinases, whose substrate phosphorylation sites often exhibit similar primary sequences. It is expected that Ca current is under a complex control of a network of protein kinases that interact and whose activities are inter-dependent. Biochemical studies have shown that several protein kinases can phosphorylate Ca channels in vitro (Sieber et al. 1987; O'Callahan & Hosey, 1988). For example, L-type channels in cardiomyocytes possibly have two modes of regulation. One site requires phoshorylation and its activation results in increased opening probability of the channel in the presence of low concentration of OA (< 1 μM) and low levels of cAMP, whereas activation of the other site decreases closing rate and requires higher levels of OA (> 10 μM) and high cAMP (Ono & Fozzard, 1993).

To determine a possibility whether Ca channels could be phosphorylated at more than one site at the same time, for instance by both PKA and another kinase (PKX) whose activity was revealed by phosphatase inhibition, additivity between the effect of phosphatase inhibitors and drugs that elevate cAMP was examined. Such experiments could provide at least some vague idea of the function of protein kinases. In the present experiments it was shown that forskolin in DR neurones increases cAMP and potentiates Ca currents. In the presence of both forskolin and OA in the

recording pipette, the effect on the enhancement of the peak Ca current was additive, that is the enhancement was stronger than when OA was present alone. It is interesting that, with a high dose of OA in the cells, cAMP-dependent stimulation of the peak current was smaller than in the absence of phosphatase inhibition. It is possible to suggest two hypotheses based on this observation. First, Ca channel can be phosphorylated on two or more different phosphorylation sites simultaneously, and phosphorylation at both sites stimulates Ca current, possibly up to a certain limit. Secondly, the channel can be phosphorylated only at a single site, and, for example, it might be that the applied doses of the phosphatase inhibitors did not completely phosphorylate that site, so that elevation of cAMP results in additional phosphorylation. In our experiments it was not possible to determine why protein phosphatase inhibitors were unable to fully phosphorylate that site. However, in support of the latter hypothesis is the slight difference in the efficacy of OA and MC in elevating the peak Ca current. Moreover, there is a possibility that some other protein phosphatase regulates the process.

Cadogan et al. (1993) showed that activation of 5-HT_{1A} receptors by 8-OH DPAT exhibited dual effect, i.e. it induced an increase in cAMP in rat cerebral cortex and in rat and guinea-pig hippocampus in vivo, and inhibited forskolin-stimulated adenylyl cyclase activity in hippocampus in vitro. It appears that this negative coupling between 5-HT_{1A} receptor stimulation and adenylate cyclase has only been observed under stimulated conditions, i.e. in the presence of forskolin (Devivo & Maayani, 1986). Forskolin has also been shown to act in a cAMP-independent fashion (Laurenza et al. 1989). There are indications to suggest that the drug can modulate voltage-dependent channels, for example K^+ channel, and inhibit an ion flow via nicotinic and GABA receptors (Laurenza et al. 1989).

As described, G-proteins are suggested to be involved in a regulation of Ca channels in several ways, including a direct or membrane-delimited G-protein-Ca channel interaction, and indirect ones that employ various second messengers. For

instance, NE modulates Ca current via G_s, that then activates cAMP-dependent protein kinase in cultured heart muscle cells (Cachelin et al. 1983; Sculptoreanu et al. 1993). There are indications to suggest PKC to underlie both potentiation (Dösemeci et al. 1988) and inhibition of Ca currents in a number of neuronal types, such as NE-induced modulation of Ca currents in chick DRG neurones (Rane & Dunlap, 1986), neuropeptide Y in rat DRG cells (Rane et al. 1989), bradykinin-induced block of Ca current in a rat DRG cell line (Boland et al. 1991) and increase in the current following application of 5-HT in *Aplysia* sensory neurones (Braha et al. 1993). Another second messenger arachidonic acid mediated the inhibition of Ca current in *Aplysia* neurones, following activation of a PTX sensitive G-protein (Brezina et al. 1987).

In contrast, in DR neurones H-7, a non-selective PKA and PKC inhibitor, and staurosporine, that is more selective for PKC, failed to prevent 8-OH DPAT-induced inhibition of the peak current. Phorbol esters and arachidonic acid were also ineffective in preventing this modulatory effect (Penington et al. 1991). Secondly, it has been shown in other cell types that transmitter-induced inhibition of Ca current is a rapid process, unlike most known second messenger modulated events (Dolphin, 1991a). Thirdly, an application of 5-HT to the extracellular solution had no effect on unitary Ca currents recorded with cell-attached patches (Penington et al. 1991), thus denying the need for a freely diffusible second messenger and supporting a membrane-delimited mechanism (Bean, 1989b; Lipscombe et al. 1989). This evidence suggests that the action of 8-OH DPAT is mediated via a membrane bound G-protein that can, without generating a second messenger, modulate Ca currents.

In support of the idea that no second messenger pathway is involved, it has been shown that PKC activation does not produce the characteristic voltage-dependent inhibition of Ca current in many cell types (Wanke et al. 1987; Plummer & Hess, 1991), furthermore, arachidonic acid had no effect in DRG cells (Dolphin et al. 1989). Extracellularly applied NE had no effect on Ca channel current in cell-attached patches, in contrast to the experiments done in similar fashion, but with the agonist

present in the patch pipette. The same observation was made following applications of NE on sympathetic ganglion neurones (Lipscombe et al. 1988a), neuropeptide Y on myenteric neurones (Hirning et al. 1988b) and baclofen on DRG cells (Green & Cottrell, 1988). However, there is evidence that NE applied from outside the patch inhibited Ca current in chick DRG neurones (Anderson & Dunlap, 1988). Thus, it has not been unequivocally shown in neuronal cells that the inhibition of Ca channels by neurotransmitters occurs by direct interaction with activated G-protein, as proposed G_s interaction occurs with Ca channels in heart and muscle cells (Yatani et al. 1987). No method so far has provided direct evidence that in neurones a second messenger is not required. Indeed, if the required second messenger is, for instance, diacylglycerol or another membrane associated compound, it would not be expected to diffuse to or from the membrane under the patch. As shown here, evidence suggests that in spite of the fact that a PTX sensitive G-protein is involved, inhibition of adenylyl cyclase is not essential for the response, which still occurs when the cAMP levels are elevated above control levels. Nevertheless, the activity of Ca channels in DR neurones, as in heart cells, is increased by cAMP-dependent phosphorylation and forskolin is able to increase the peak Ca current in DR neurones. Unequivocal evidence will only come from systems in which cloned receptors, G-proteins and Ca channels are reconstituted into lipid bilayers.

Conclusions

In this study, it was shown that 5-HT_{1A} receptor activation modulates HVA Ca current, and the effects are probably G-protein mediated. 8-OH DPAT produced a slowing of activation and reduction of the current amplitude in DR neurones. It is possible that these two effects are voltage-dependent and voltage-independent,

respectively. These effects may be mediated via different G-proteins, that are both PTX sensitive. It is also possible that the ligand activates one G-protein type which then activates one or more different second messengers. On the other hand, it might be that the agonist interacts with a heterogenous collection of Ca channels, for example more than one N-type channel each with different properties, as shown by Williams et al. (1992) in ω-CgTx sensitive N-type Ca channels in human tissue. Functional consequences of an existence of voltage-dependent and voltage-independent action of 8-OH DPAT in DR cells are very broad. For example, since Ca entry via HVA channels normally occurs during a relatively short period of time, it may well be that slowing of activation has a greater influence on the increase in intracellular calcium, than would a direct reduction in the current amplitude (Penington et al. 1992). The existence of multiple modulatory components could allow fine tuning of Ca-dependent processes, during various physiological functions. The co-existence of both voltage-dependent and voltage-independent inhibitory mechanisms might possibly provide for time-dependent changes in cellular functions. Kinetic-slowing component, that is voltage-dependent, would be phasic in nature, such as in repetitive firing, and attenuated with subsequent stimulation. A reduction in the current amplitude, that is voltage-independent, would be tonic in nature and persist longer, even during high-frequency stimulation. Such dual mechanism of transmitter action could be well suited for DR neurones, that are known to exhibit a slow and regular pattern of action potentials, in addition to regulation by serotonin release, that is probably mediated via the 5-HT_{1A} receptor.

It is possible that modulation of Ca currents in DR neurones results from a direct action of G-proteins on Ca channels. However, presence of a second messenger pathway can not be excluded. Indeed, it might be an advantage to have several modulatory pathways acting simultaneously with different time courses. One pathway might serve and permit immediate changes in cell processes, and the other one could possibly integrate signals over a relatively longer period of time. In that way, the

pathways can involve various receptor subtypes, some of them closely located to the sources of rapid transmitter release and others more diffusely spread over the cell surface. The membrane-delimited pathway can probably affect only nearby Ca channels, whereas the diffusible messenger pathway may act more broadly. Different pathways may not necessarily be under the same control, and that would lead to a greater variety of responses, and perhaps more subtle control. In support of this view it is known that more than one pathway can be selectively activated by a single agonist. For instance, in chick ciliary ganglion neurones somatostatin inhibited Ca current and slowed the activation kinetics in whole-cell, but not in a perforated patch recordings (Meriney et al. 1994). The effect observed in a whole-cell recordings is similar to the effect of other neurotransmitters on Ca current and might present a membrane-delimited pathway. Inhibition of Ca current with no change in activation kinetics is mediated via a second messenger pathway involving cyclic GMP-dependent protein kinase (cGMP-PK). This suggests that activation of the somatostatin receptor initiates two cascades, both acting via G-proteins. This effect can possibly occur through two different types of G-proteins or via a single G-protein that triggers two different effectors, as demonstrated for G_s in cardiac myocytes (Yatani et al. 1987; Mattera et al. 1989).

Protein phosphatase 1, 2A and 2B inhibitors enhanced the peak Ca current in 80% of neurones tested, and, in addition, prevented fast "run-down" of Ca current in DR neurones. However, they had no effect on the reduction of the peak current and slowing of the activation kinetics caused by the external application of 8-OH DPAT. This suggests that phosphorylation is not directly involved in the modulatory effect of 8-OH DPAT on the HVA currents in DR neurones and does not share the same pathway of modulation with 8-OH DPAT. This fact also argues against a role for second messengers known to modify Ca channel function via phosphorylation and/or dephosphorylation, in the action of 8-OH DPAT.

Appendix A

Appendix A: List of abbreviations used in the text.

1-NO	= 1-norokadaone
2-CA	= 2-chloroadenosine
4-AP	= 4-aminopyridine
5-HT	= 5-hydroxytryptamine
8-OH DPAT	= 8-hydroxy-2-(di-n-propylamino)tetraline
ATP	= adenosine triphosphate
BAPTA	= 1,2-bis(2-aminophenoxy)ethane N,N,N′,N′-tetraacetic acid
Ca (Ca^{2+})	= calcium ion
cAMP	= cyclic adenosine monophosphate
Cd^{2+}	= cadmium ion
cGMP	= cyclic guanosine monophosphate
CNS	= central nervous system
DADLE	= D-Ala, D-Lue-enkephalin
DHP	= dihydropyridine
DMF	= dimethyl-formamide
DR	= dorsal raphé
DRG	= dorsal root ganglion
EGTA	= ethylene-glycol-bis(β-aminoethyl-ether)-N,N,N′,N′-tetraacetic acid
FKBP	= FK binding protein
FTX	= funnel web spider toxin
GABA	= γ-aminobutyric acid
GDP-β-S	= guanosine 5′-O-2-thiodiphosphate
GTP	= guanosine triphosphate

GTP-γ-S	= guanosine 5′-O-3-thiotriphosphate
HEPES	= N-2-hydroxyethyl-piperazine-N′-2-ethanesulphonic acid
HVA	= high-voltage activated Ca current
LSD	= D-lysergic acid diethylamide
LVA	= low-voltage activated Ca current
MC	= microcystin-LR
NAN 190	= 1-(2-methoxyphenyl)-4-[4-(2-phthalimmidobutyl] piperazine
NE	= norepinephrine
NPY	= neuropeptide Y
OA	= okadaic acid
PIPES	= piperazine-N,N′-bis[2-ethane-sulfonic acid]
PKA	= protein kinase A
PKC	= protein kinase C
PP	= protein phosphatase
PTX	= pertussis toxin
SEM	= standard error of the mean
SEVC	= single-electrode voltage-clamp
TEA	= tetraethylammonium
Trizma	= 2-amino-2-hydroxymethyl-1,3-propandiol
TTX	= tetrodotoxin
V_H	= holding potential
ω-CgTx	= ω-conotoxin GVIA

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